Species Differences of Serum Albumins: I. Drug Binding Sites

Takamitsu Kosa,[1] Toru Maruyama,[1,2] and Masaki Otagiri[1,3]

Received March 24, 1997; accepted July 4, 1997

Purpose. The purpose of this study was the classification and identification of drug binding sites on albumins from several species in order to understand species differences of both drug binding properties and drug interaction on protein binding.

Methods. Binding properties and types of drug-drug interaction on the different albumins were examined using typical site I binding drugs, warfarin (WF) and phenylbutazone (PBZ), and site II binding drugs, ibuprofen (IP) and diazepam (DZ) on human albumin. Equilibrium dialysis was carried out for two drugs and the free concentrations of drugs were then treated using the methods of Kragh-Hansen (*Mol. Pharmacol.* **34**. 160–171, (1988)).

Results. Binding affinities of site I drugs to bovine, rabbit and rat albumins were reasonably similar to human albumin. However, interestingly, those to dog albumin were considerably smaller than human albumin. On the other hand, binding parameters of DZ to bovine, rabbit and rat albumins were apparently different from those of human albumin. These differences are best explained by microenvironmental changes in the binding sites resulting from change of size and/or hydrophobicity of the binding pocket, rather than a variation in amino acid residues.

Conclusions. We will propose herein that mammalian serum albumins used in this study contain specific drug binding sites: Rabbit and rat albumins contain a drug binding site, corresponding to site I on human albumin, and dog albumin contains a specific drug binding site corresponding to site II on the human albumin molecule.

KEY WORDS: drug binding site; human serum albumin; experimental animal serum albumins; species difference.

INTRODUCTION

Albumin is the most abundant protein in mammalian systems, and plays an important role in the transport and deposition of a variety of endogenous and exogenous substances in blood (1,2). It is generally accepted that there are two major specific drug binding sites, site I and site II on human albumin (3). X-ray studies of crystalline human albumin (4) support this view and indicate that site I and site II are located within specialized cavities in subdomain IIA and IIIA, respectively. In addition, the crystallographic structure of equine albumin has also been determined, and the data suggests that there are also two specific drug binding sites on this molecule (5) as well. This suggests that, with respect to binding sites, other mammalian albumins are analogous to human albumin, considering the structural similarities between the molecules. In fact, Panjehshahin proposed the hypothesis that bovine, dog, horse and sheep albumins contain binding sites for warfarin (WF) and dansylsarcosine, which have properties similar to site I and site II on human albumin (6). This group also implied that rat albumin contains the binding sites different from other albumins (6). This idea, however remains open to question, since they did not measure the free concentration of the fluorescent probes and carry out mutual displacement experiments in their study. If a more detailed characterization of drug binding sites, such as the location and drug binding properties were carried out, this would allow more valid comparisons of drug-drug interactions to be made between animal vis-a-vis human albumins. The present study was undertaken to investigate whether or not animal albumins, including those from rat and dog contain drug binding sites which are equivalent to site I and site II of human albumin. This was carried out by employing WF, phenylbutazone (PBZ), ibuprofen (IP) and diazepam (DZ), as marker ligands, which are known to specifically bind to site I and site II on the human albumin molecule. The study was conducted using rigorous competition experiments, involving all possible combinations of the four marker ligands for four animal albumins, and the data compared to that for human albumin.

MATERIALS AND METHODS

Reagents

Human albumin was donated by the Chemo-Sera-Therapeutic Research Institute (Kumamoto Japan). Bovine, dog, rabbit and rat albumin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). The samples were defatted with activated charcoal in solution at 0°C, acidified with H_2SO_4 to pH 3 and then freeze-dried (7). All albumins used in this study showed only one band by SDS-PAGE, and the molecular masses were assumed to be about 66 kDa. Potassium WF (Eisai Co., Tokyo, Japan), IP (Kaken Pharmaceutical Co., Tokyo, Japan) and DZ (Sumitomo Pharmaceutical Co., Osaka, Japan) were obtained from the manufactures and PBZ was purchased from Nakalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade. 0.067 M Phosphate buffer (pH 7.4) was prepared with dibasic sodium phosphate and monobasic sodium phosphate and used exclusively in this study.

Determination of Binding Parameters

In order to quantitatively analyze the binding mode, binding parameters were determined by equilibrium dialysis. Aliquots (1.5 ml) of ligand-albumin mixture at various ratios were placed in plastic dialysis cells (Sanko, Fukuoka, Japan), whose compartments were separated by Visking cellulose membranes (12 kDa molecular weight cut off), and dialyzed against the same volume of buffer at 25°C for 12 h. After equilibrium was obtained, ligand concentrations as the free ligand (C_f) were determined by HPLC.

The HPLC system consisted of a Hitachi 655A-11 pump and a Hitachi L-4000 type UV detector or Hitachi L-7480 type fluorescence detector. An Inertsil ODS-2 column (5 μM, 4.6 × 150 mm) was used as the stationary phase. The mobile phase consisted of 0.1 M acetate buffer (pH 4.5)-acetonitrile (40:60, v/v) for WF, PBZ and IP, and water-acetonitrile (40:60, v/v)

[1] Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oehonmachi, Kumamoto 862, Japan.

[2] Department of Pharmacy, Japanese Red Cross Kumamoto Hospital, 2-2-1 Nagamine-Minami, Kumamoto 862, Japan.

[3] To whom correspondence should be addressed. (e-mail: otagirim@gpo.kumamoto-u.ac.jp)

0724-8741/97/1100-1607$12.50/0

for DZ. PBZ and DZ were detected at 270 nm and 230 nm using a UV monitor and WF and IP using a fluorescence monitor. The excitation/emission wavelengths were 320 nm/390 nm and 263 nm/293 nm for WF and IP, respectively.

Binding parameters were determined by fitting the experimental data to the following Scatchard equation using a nonlinear squares program (MULTI program).

$$r = \sum_{i=1}^{m} \frac{n_i K_i C_f}{1 + K_i C_f} \quad (1)$$

where n_i is the number of binding sites and K_i is the association constant in the ith binding class, and r is the mole of bound ligand per mole of total protein (C_b/P_t).

Interaction Mode of Two Ligands at High-Affinity Binding Sites on Various Serum Albumins

In order to simultaneously estimate the interaction mode between two ligands binding to each primary binding site of albumin, the data were treated according to the method of Kragh-Hansen (8). According to this method, a coupling constant (χ) is represented by $\chi = K_{BA}/K_A$ or $\chi = K_{AB}/K_B$, which is capable of distinguishing interaction manner. K_{BA} is the association constant of ligand A in the presence of ligand B, and K_{AB} is that of ligand B in the presence of ligand A. If A and B bind independently to albumin, then $\chi = 1$; i.e., K_{BA} is equal to K_A and K_{AB} to K_B. $\chi > 1$ and $0 < \chi < 1$ indicate cooperative and anti-cooperative binding, respectively. In addition, competitive displacement between two ligands is represented by $\chi = 0$. The χ value was determined as described previously (8).

RESULTS

Determination of Binding Parameters of Four Drugs to Different Albumins

In order to clarify the species differences of the interaction between the drugs and various albumins, typical site I binding drugs for human albumin, namely, WF and PBZ, and site II binding drugs, IP and DZ, were used as model drugs. Fig. 1 shows scatchard plots of the binding of IP to different albumins as a typical example, and Table 1 shows the obtained binding parameters of WF and PBZ to different albumins. The binding constant of WF to rat albumin was the highest, and those to bovine and rabbit albumins were nearly the same as for human albumin. Interestingly, binding parameters for the primary binding site of WF and PB to dog albumin were not obtained, as opposed to other albumins. In addition, the binding constants of PBZ to rabbit and rat albumins were low, compared with human and bovine albumins.

The binding parameters for site II drugs, IP and DZ, are shown in Table 2. For IP, the numbers of the primary binding site to all albumins were 1, but the numbers for secondary binding site were different depending upon albumin species. The primary binding constants to human and dog albumins were relatively large, compared with rabbit and rat albumins. However, the binding parameters for the primary binding site of DZ for bovine, rabbit and rat albumins were not determined, different from those to human and dog albumins.

Numerous reports have appeared relative to the binding parameters of these drugs to different serum albumins, especially human albumin. The binding parameters listed in Tables 1 and 2 are generally in good agreement with the values reported by many (but not all) of these reports (9–14). The discrepancies may be due to the differences in experimental conditions, such as the albumin lot (difference in fatty acid content and mercapto albumin content), pH, temperature and salt concentration.

Interaction Between WF, PBZ, IP and DZ Bound to High Affinity Binding Sites on Albumin Molecules

For identification of drug binding sites for each drug, we examined the interaction of two ligands. Firstly, the interaction between WF and PBZ, site I binding drugs on human, was investigated for the animal serum albumins. Fig. 2 shows the interaction mode of these two ligands on albumins. It is clear from Fig. 2 that WF interacted with PBZ on human albumin in a competitive manner, as has been previously reported (15). A similar phenomenon was observed for rabbit and rat albumins. For the case of bovine albumin, the type of interaction was different from that of human, rabbit and rat albumins. In these cases, the displacements appeared to be anti-cooperative. On the other hand, the interaction mode of WF- and PBZ on dog albumin was not determined because of the lacking of primary binding parameters for WF and PBZ.

Fig. 3 shows interactions of WF or PBZ with IP, specific ligands, which are known to bind to site I and site II on human albumin. As shown in Fig. 3, the interaction of WF and IP with rat albumin appeared to be independent, as expected from the results of human albumin. For the case of the PBZ-IP system, the type of interaction was anti-cooperative, because binding curves constructed using a common value of $\chi = 0.58 \pm 0.02$ agreed with the experimental finding. In addition, both interactions of WF-IP or PBZ-IP systems for rabbit albumin showed anti-cooperative binding. However, no mutual displacements for WF-IP or PBZ-IP were observed in bovine albumin. All the displacement data obtained are summarized in Table 3.

DISCUSSION

Although serum albumins are highly homologous, in terms of amino acid sequences (about 80% between human and the species used in this study), drug binding properties differ considerably among the species. As a result, species differences with respect to drug binding to serum albumins have been extensively examined (16–19). However, since these studies were not investigated systematically in terms of drug binding sites, the issue of whether or not there are specific drug binding sites on serum albumins is not clear, nor is the issue of the nature of such sites.

On the other hand, the highly homologous amino acid sequences of serum albumins of other species are useful in studies of active amino acid residues, as "mimics" of human albumin. This is especially useful in the comparison of structures and ligands binding properties among the species which may be useful in determining the functionality of certain amino acid residues.

For the purpose of obtaining information, we attempted to identify drug binding sites on different albumins using typical site I binding drugs on human albumin (WF for the WF binding site and PBZ for the azapropazone binding site), as well as typical site II binding drugs for human albumin (IP and DZ).

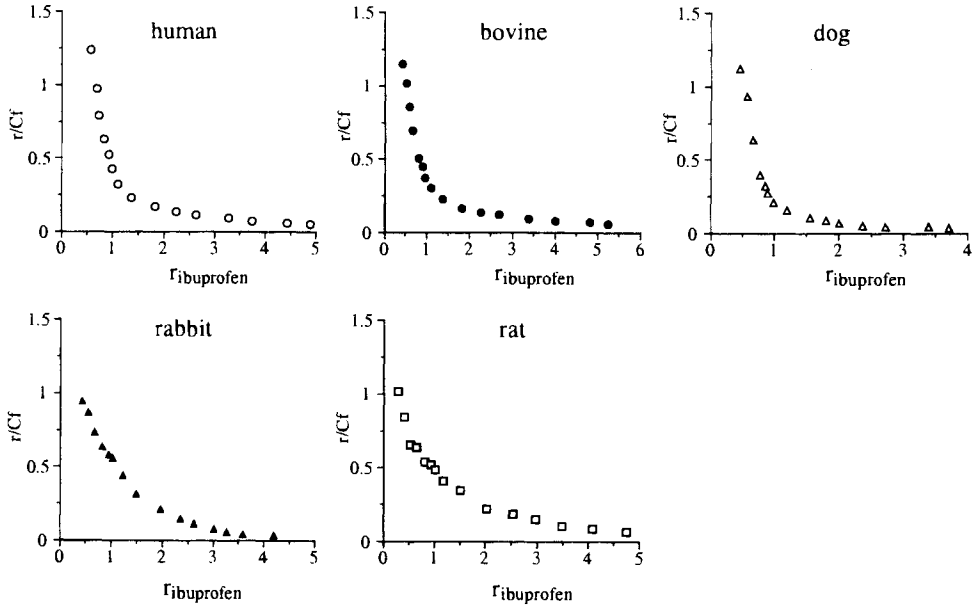

Fig. 1. Scatchard plots of the binding of IP to different albumins (20 μM).

Table 1. Binding Parameters of Warfarin and Phenylbutazone (Site I on Human Albumin Binding Drug) to Different Serum Albumins Estimated by Equilibrium Dialysis at pH 7.4 and 25°C

	Warfarin				Phenylbutazone			
	n_1	K_1 ($\times 10^5$ M^{-1})	n_2	K_2 ($\times 10^4$ M^{-1})	n_1	K_1 ($\times 10^5$ M^{-1})	n_2	K_2 ($\times 10^4$ M^{-1})
human	1	3.04 ± 0.34	2	2.92 ± 0.34	1	11.37 ± 0.89	2	1.62 ± 0.42
bovine	1	2.65 ± 0.44	2	2.02 ± 0.35	1	17.63 ± 3.87	3	1.38 ± 0.39
dog	N.D.	N.D.	2	0.23 ± 0.05	N.D.	N.D.	2	0.39 ± 0.05
rabbit	1	1.82 ± 0.15	2	1.29 ± 0.05	1	3.39 ± 0.52	2	3.24 ± 0.63
rat	1	7.01 ± 1.53	4	0.86 ± 0.23	1	5.59 ± 0.95	3	0.62 ± 0.11

Note: The values represent the mean ± S.D. N.D.; Not determined.

As demonstrated from the analysis of the X-ray crystallographic structure of human albumin, site I and site II are located in subdomain IIA and subdomain IIIA, respectively (4). The three-dimensional structures of albumins appear to be roughly similar among the species, since equine albumin, is about 80% homologous with human albumin, possessed almost similar X-ray crystallographic structure to human albumin (5).

Site I binding drugs, which are generally bulky heterocyclic molecules, containing a widely delocalized negative charge (9), are thought to bind to human albumin by mainly hydrophobic interactions. Static interactions with ^{199}Lys and ^{242}His (4) should also be considered. Furthermore, ^{214}Trp on site I, the only tryptophan residue on human albumin, is responsible for the formation of the IIA binding site by limiting solvent accessi-

Table 2. Binding Parameters of Ibuprofen and Diazepam (Site II on Human Albumin Binding Drug) to Different Serum Albumins Estimated by Equilibrium Dialysis at pH 7.4 and 25°C

	Ibuprofen				Diazepam			
	n_1	K_1 ($\times 10^5$ M^{-1})	n_2	K_2 ($\times 10^4$ M^{-1})	n_1	K_1 ($\times 10^5$ M^{-1})	n_2	K_2 $\times 10^4$ M^{-1})
human	1	35.63 ± 3.25	6	1.78 ± 0.49	1	17.49 ± 6.26	2	1.78 ± 0.16
bovine	1	22.54 ± 1.54	8	1.36 ± 0.09	N.D.	N.D.	2	0.16 ± 0.01
dog	1	31.52 ± 1.76	3	2.46 ± 0.17	1	8.06 ± 0.57	2	2.13 ± 0.34
rabbit	1	15.88 ± 2.38	3	4.08 ± 0.52	N.D.	N.D.	2	0.15 ± 0.04
rat	1	10.91 ± 0.67	5	3.34 ± 0.48	N.D.	N.D.	2	0.06 ± 0.02

Note: The values represent the mean ± S.D. N.D.; Not determined.

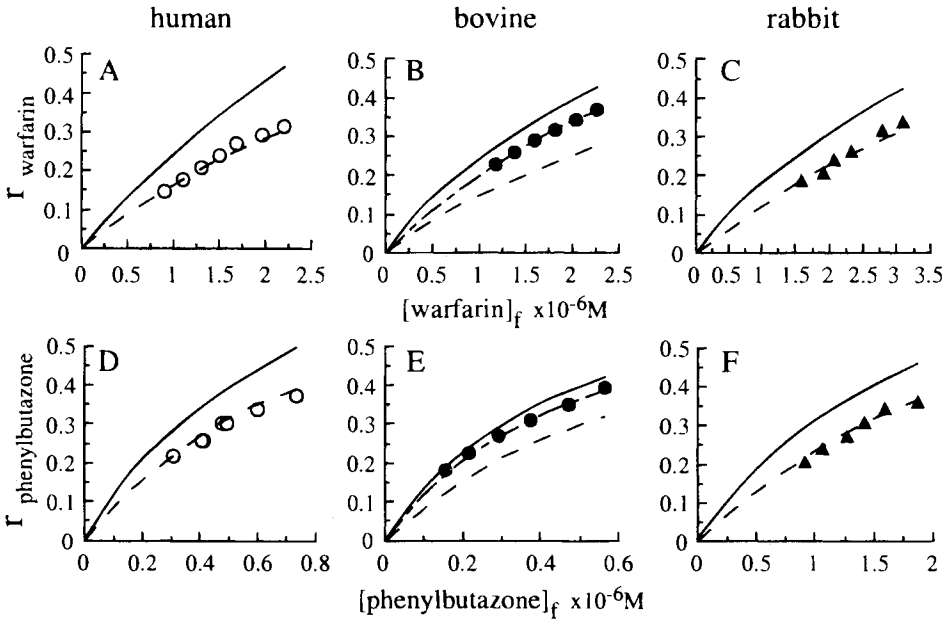

Fig. 2. WF binding to different albumins (20 μM) in the presence of PBZ (A–C), and, PBZ binding in the presence of WF (D–F) at pH 7.4 and 25°C. WF(5–11 μM) (A), or PBZ (5–9 μM) (D), binding to human albumin in the presence of PBZ (9 μM) or WF (11 μM), respectively. WF(6–12 μM) (B), or PBZ (4–9 μM) (E), binding to bovine albumin in the presence of PBZ (9 μM) or WF (12 μM), respectively. WF (7–13 μM) (C), or PBZ (6–11 μM) (F), binding to rabbit albumin in the presence of PBZ (11 μM) or WF(13 μM), respectively. (—); Theoretical curves, assuming the independent binding of two ligands surmised by binding parameters, n_1 and K_1. (---); Theoretical curves postulating the competitive binding of two ligands on high-affinity binding sites of each albumins. (-·-·); Theoretical curves assuming binding of WF and PBZ to different high-affinity binding sites on bovine albumin with mutual interaction.

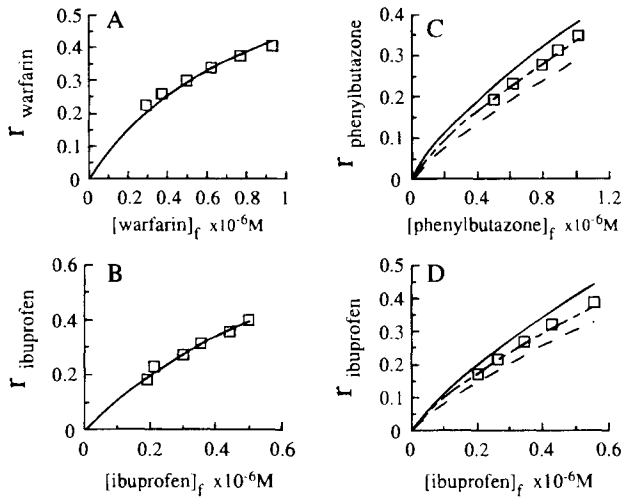

Fig. 3. WF (5–10 μM) (A) and PBZ (5–9 μM) (C) binding in the presence of IP (4–8 μM), and IP (4–9 μM) binding in the presence of WF (10 μM) (B) and PBZ (10 μM) (D) to rat albumin at pH 7.4 and 25°C. (—); Theoretical curve, assuming the independent binding of two ligands surmised by binding parameters, n_1 and K_1. (---); Theoretical curve postulating competitive binding of two ligands on high-affinity binding sites of each albumins. (-·-·); Theoretical curve postulating that WF and IP have different high-affinity binding sites on rabbit, which mutually interact.

Table 3. Interaction Manner of Two Drugs on Different Albumins

Ligands	Human	Bovine	Dog	Rabbit	Rat
WF-PBZ	C	AC	—	C	C
WF-IP	I	I	—	AC	I
WF-DZ	I	—	—	—	—
PBZ-IP	AC	I	—	AC	AC
PBZ-DZ	AC	—	—	—	—
IP-DZ	C	—	C	—	—

Note: The letters represent as follows: C; competitive, AC; anti-cooperative, I; independent. —, represent not examined. The abbreviations of ligands are follows: WF; warfarin, PBZ; phenylbutazone, IP; ibuprofen, DZ; diazepam.

bility (4). Interestingly, as shown in Table 1, the binding parameters for site I drugs to dog albumin are significantly different from those to other species. This difference might be due to the small difference of microenvironment in the three dimensional structures related to the binding of site I drugs such as hydrophobicity and electric charge between dog albumin and other albumins. In the case of dog albumin, a defect might exist at the location corresponding to site I. However, amino acid residues, ^{199}Lys, ^{242}His, ^{214}Trp and hydrophobic residues (Leu, Ile, Pro, Val and Ala) near these residues, responsible for ligand binding to site I are nearly conserved in dog albumin (2). Therefore, these interesting phenomena might be due to slight or a possibly

considerable alteration in tertiary structure near this binding site on the dog albumin molecule.

Numerous drugs are also bound to site II on human albumin, and these drugs contain some common structural features. One of these is the presence of aromatic carboxylic acids, whose negative charge is isolated from the nonpolar region of the molecule (9), for example, IP, naproxen, mefenamic acid, and related molecules. The others are diazepines, such as DZ, oxazepam and nimetazepam.

As shown in Table 2, the binding parameters of DZ to bovine, rabbit and rat albumins were remarkably different: the primary binding parameters were not obtained. This observation may reflect, to some extent, differences in the microenvironment of the drug binding site on these albumins, corresponding to site II on human albumin. According to the X-ray study of crystalline human albumin, the carboxylate of triiodobenzoic acid interacts primarily with ^{410}Arg and is within 4.0 Å of the oxygen of ^{411}Tyr, and the aromatic ring of triiodobenzoic acid interacts with the hydrophobic residues, e.g., Leu, Ile, Pro, Val and Ala (4). The carboxyl groups of site II binding drugs play an important role in binding to this site, since the absence of a carboxyl group changes the site II binding site to a site I binding site, for molecules such as suprofen (20). However, ^{410}Arg and ^{411}Tyr are conserved among all species used in this study, and hydrophobic residues of these albumins are also well conserved.

A previous study showed that the driving forces for DZ binding to human albumin is due mainly to hydrophobic interaction and a partial involvement of the molecular size of the ligands (10). As described above, hydrophobic residues, which are thought necessary for the binding of DZ, near the binding site called site II on human albumin are well conserved among the species. Wanwimolruk *et al.* (21) reported that human albumin site II is a hydrophobic cleft, approximately 16 Å deep and 8 Å wide, with a cationic group located near the surface. These facts suggest that the hydrophobic interactions between DZ and bovine, rabbit and rat albumins are weakened, as the result of a tertiary structure change in cavity size rather than the loss of hydrophobic residues. As above mentioned, important amino acid residues for binding at site II are nearly completely conserved among the species, although only one amino acid residue, ^{195}Lys, related to the ligand binding to site II (22), differs among the species. In this case, Lys for human and dog albumins is substituted with Arg for bovine and rat albumins, and with Gln for rabbit albumin. Of course, it is too early to postulate the essential amino acid residue at the binding for site II, but it is possible that one or more of these amino acid residues are essential for binding. The amino acid residues involved drug binding sites will be elucidated by applications of genetic engineering, such as site-directed mutagenesis, and this work is underway at this laboratory.

As shown in Table 3, the interaction mode between WF and PBZ was nearly the same for human, rabbit and rat albumins. This suggests that the specific drug binding site, like site I on human albumin, was probably constructed on these albumins. On the other hand, the interaction between WF and PBZ on bovine albumin did not show competitive characteristics. Fehske *et al.* (14) reported that site I on human albumin was created with two regions, a WF binding site and an azapropazone binding site. These regions are known to overlap on another on human albumin. It is likely that the WF region is distinct from the azapropazone region on bovine albumin, compared with on human albumin. Furthermore, a recent study reports that site I on human albumin consists of at least three regions, Ia, Ib and Ic(15). As a result of these studies, the drug binding site on bovine albumin, which corresponds to site I on human albumin may not be as complicated as previously thought.

The characteristics of the interaction between WF and IP to bovine and rat albumins, and between PBZ and IP to rabbit and rat albumins were similar to human albumin. However, the interactions between WF and IP to rabbit albumin and between PBZ and IP to bovine albumin were somewhat different from human albumin. These differences may indicate that the WF binding site is located in relatively near to the location of the IP binding site and that they may affect each other anticooperatively for the case of rabbit albumin. The PBZ binding site appears to be completely separate from the IP binding site of bovine albumin. Anyway, since these interaction manners are rather similar to the cases of human albumin, it can be concluded that specific drug binding sites (resembling those on humans) probably exist on bovine, rabbit and rat albumins, although they are somewhat different from the drug binding sites on human albumin.

Interactions between site II binding drugs, IP and DZ, on dog albumin are quite similar to those on human albumin. With respect to site II, both the binding constant and characteristics of the interaction of IP and DZ to dog albumin are similar to human albumin, suggesting that dog albumin contains the structures, which the other species are lacking, specifically to bind benzodiazepines, including DZ.

Fig. 4. shows possible models of the binding sites on different albumins, based on data in Table 3. Unimportant binding, observed in the binding of both WF and PBZ to dog albumin, and of DZ to bovine, rabbit and rat albumins, were omitted in these cases. Human albumin site I appears to exist for rabbit and rat albumins, but not bovine and, especially, dog albumin. However, bovine, rabbit and rat albumins are lacking in the binding sites of diazepines, and, thus are different from human albumin. Interestingly, the architecture of site II on dog albumin is quite similar to human albumin, although dog albumin lacks some of the important structures of site I.

If displacement of the protein (albumin) binding significantly affects the drug-drug interactions, binding affinity as

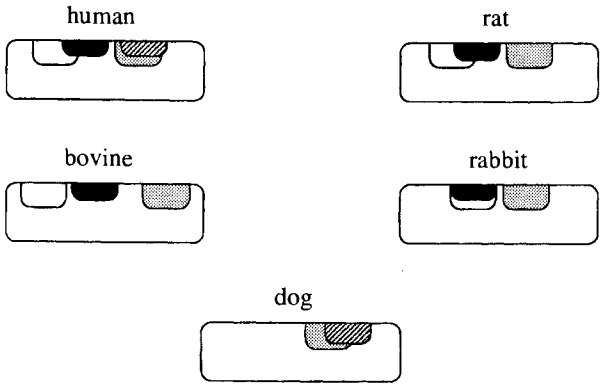

Fig. 4. Proposed model of drug binding sites on different albumins. ○; WF binding site, ■; PBZ binding site, ▨; IP binding site, ▨; DZ binding site.

well as the locations of drug binding sites are important factors of the interactions. We suggest from the results of this study that rabbit and rat should be selected for estimation of extrapolation from animal to human, and dog should be avoided with regard to drug-drug interactions on site I, but dog is the most suitable with regard to that on site II.

As pointed out by Lin (23), it is important to address the question of whether animal data can be extrapolated to humans, including protein binding characteristics. Although it is still early to conclude from these limited data on drug binding sites for different albumins, the present results provide useful, basic information on such extrapolations.

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