

The Influence of Lactic Acid on the Serum Protein Binding of Bupivacaine: Species Differences

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Various animal models have been used for studies of bupivacaine cardiovascular toxicity. These studies are difficult to relate to the clinical situation, since the disposition of bupivacaine in the various species is unknown. The serum protein binding of bupivacaine, therefore, was determined in human, sheep, monkey, dog, and rat at physiologic pH using ultrafiltration. Since a mixed acidosis results during a systemic toxicity reaction to bupivacaine, the influences of an acidic pH, resulting from the addition of lactic acid, also was examined. All sera exhibited two classes of binding sites, a high-affinity, low-capacity class (class 1) and a low-affinity, high-capacity class (class 2). When compared to human serum at physiologic pH, a significantly higher ($P < 0.05$) affinity constant for the class 1 sites was observed for all species studied, with the exception of the rat. All species studied exhibited a significantly lower ($P < 0.05$) capacity for the class 1 sites. The binding parameters of the class 2 sites displayed no significant difference. An acid pH resulted in a decrease in bupivacaine protein binding over the entire concentration range studied for all species, with the exception of the monkey. Monkey serum exhibited no change in bupivacaine binding with a decrease in pH. Since protein binding explains only a portion of the total disposition of bupivacaine, further delineation of each animal model under both acidotic and physiologic conditions needs to be accomplished before the animal studies currently under investigation can be extrapolated to the clinical situation. (Key words: Acid—base equilibrium: protein binding. Anesthetics, local: bupivacaine. Protein: binding.)

CARDIOVASCULAR TOXICITY STUDIES of bupivacaine in various animal models have been reported recently.^{1,2}

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The findings of these studies, however, are difficult to compare with those for humans since disposition of bupivacaine in these species is unknown. One of the major determinants of drug disposition is protein binding. The influence of protein binding on the activity and toxicity of local anesthetics, as well as on the primary pharmacokinetic parameters in humans, has been reported previously.³⁻⁵

It has also been reported, in the clinical situation, that a mixed acidosis results during a systemic toxicity reaction to bupivacaine.⁶ The influence of an acidic pH on the protein binding of bupivacaine, therefore, needs to be considered. *In vitro* protein binding studies of other local anesthetics have been concerned with the effect of acidosis using either CO₂ gas (respiratory acidosis) or HCl (non-respiratory acidosis) for the adjustment of serum or plasma pH.^{7,8} Lactic acid also has been studied, *in vivo*, to assess its influence on the lidocaine central nervous system toxicity threshold in monkeys.⁹ Lactic acid was used to simulate the metabolic acidosis that occurs in that species. Since lactic acid has not been examined for its effect on *in vitro* bupivacaine protein binding and since it would most likely be released *in vivo* as a result of hypoxia,^{10,11} contributing to the metabolic component of the mixed acidotic state, lactic acid was used as the acidifying agent in this study.

The binding of a drug to a protein is described quantitatively by the sum of drug binding to various classes of binding sites. The characteristics of each class of sites are described by two parameters: affinity constant (K_i) and binding capacity (n_iP_i). The K_i describes the tightness of the binding of a drug to a class of binding sites. This parameter is sensitive to alterations in protein conformation (three-dimensional structure) due to various conditions (pH, ionic strength, temperature, etc.). The n_iP_i is a description of the number of sites of a specific class available for binding. This parameter is dependent on the concentration of protein (P_i) and the number of binding sites per molecule of protein (n_i). The n_iP_i can be altered due to disease states.¹² Considering the protein binding of a drug in serum or plasma, a definition of the number of classes of sites is important in describing the mechanism of binding. The effect of a variation in a condition (*i.e.*, pH) on protein binding can be quantitated as to which class(es) of binding sites are altered. Changes in the K_i and/or n_iP_i of a class of binding sites greatly

can influence the amount of bound drug, therefore, altering the free drug concentration. It is the change in free drug concentration that affects pharmacologic action and disposition of the drug.

The purpose of this investigation was to determine the serum protein binding of bupivacaine in human, monkey, sheep, dog, and rat at their respective physiologic pH. The influence of a moderately acidic pH, caused by the addition of lactic acid, on the protein binding of bupivacaine in each species also was examined.

Methods

Serum was obtained from mature dogs and sheep of either sex and mixed breed. Rat and monkey serum was obtained respectively from mature Sprague-Dawley rats or rhesus monkeys of either sex. Human serum was obtained from venous blood of normal individuals of either sex. The drawing of human blood was approved by the Committee on Human Research. All sera were pooled respectively, separated into 10 ml aliquots, and stored frozen (-20°C) until used.

ULTRAFILTRATION

Determination of protein binding in all sera was performed by using an Amicon Micropartition System[®] (Amicon Corporation, Danvers, Massachusetts) equipped with a YMT membrane (Amicon). Preliminary experiments have determined that the YMT membrane did not exhibit any nonspecific adsorption of bupivacaine.

Serum protein binding was determined at bupivacaine concentrations of 1, 3, 5, 7, 10, 20, 40, 60, and 100 $\mu\text{g}/\text{ml}$. Four replications of each concentration were completed at each pH. All binding experiments were conducted at the physiologic pH for each species and at a pH representing moderate acidosis (0.4 units lower than normal). The pHs examined were: human, 7.40 and 7.00;

monkey, 7.40 and 7.00; sheep, 7.44 and 7.04; dog, 7.36 and 6.96; and rat, 7.40 and 7.00.

The sera containing the desired concentration of bupivacaine were adjusted to physiologic pH using 1 N HCl or NaOH and allowed to equilibrate at room temperature with mixing for 1 h. The total concentration of bupivacaine contained in the sera was determined by gas chromatography at the time of ultrafiltration.

Ultrafiltration was accomplished by subjecting 1 ml of serum to centrifugation at $2,050 \times g$ for 40 min at 30°C using a clinical centrifuge (International Equipment Company, Needham, Massachusetts) equipped with a Model 803 angle head centrifuge rotor (IEC). This method resulted in the generation of approximately 0.5 ml of ultrafiltrate. It has been reported that the free serum concentration of a drug as determined by ultrafiltration is independent of the fraction of sample filtered.¹³ Following centrifugation, the ultrafiltrate was removed for analysis.

The remaining sera of the 10-ml aliquot then were adjusted to the appropriate acidic pH using lactic acid USP (Baker Chemical Company, Phillipsburg, New Jersey). The sera then were treated as above for the determination of protein binding at acidic pH.

ANALYSIS

Bupivacaine concentrations were determined by gas chromatography using a modification of the method of Mather and Tucker.¹⁴ A Hewlett Packard Model 5840[®] gas chromatograph (Hewlett Packard, Avondale, Pennsylvania), equipped with a six-foot glass column packed with 3% OV-17 on Gas Chrom Q and a nitrogen-specific detector was used. Calibration curves were constructed from standard concentrations of bupivacaine analyzed with each set of samples. The coefficient of variation of the assay method is 2–5% at 5 $\mu\text{g}/\text{ml}$ and 12–15% at 10 $\mu\text{g}/\text{ml}$.

TABLE 1. Serum Binding Affinities and Capacities at Physiologic pH*

Species	$n_1 P_1$ (M)	K_1 (M^{-1})	$n_2 P_2$ (M)	K_2 (M^{-1})
Human	1.56×10^{-5} $\pm 1.4 \times 10^{-6}$	1.69×10^6 $\pm 2.83 \times 10^5$	3.71×10^{-4} $\pm 1.07 \times 10^{-4}$	4.21×10^3 $\pm 1.64 \times 10^3$
Sheep	$5.64 \times 10^{-6}\dagger$ $\pm 6.49 \times 10^{-7}$	$1.27 \times 10^7\dagger$ $\pm 2.96 \times 10^6$	2.74×10^{-4} $\pm 4.51 \times 10^{-5}$	8.25×10^3 $\pm 1.90 \times 10^3$
Monkey	$4.10 \times 10^{-6}\dagger$ $\pm 2.29 \times 10^{-7}$	$1.29 \times 10^7\dagger$ $\pm 4.80 \times 10^6$	9.13×10^{-4} $\pm 2.94 \times 10^{-4}$	1.04×10^3 $\pm 3.60 \times 10^2$
Rat	$3.91 \times 10^{-6}\dagger$ $\pm 6.55 \times 10^{-7}$	2.21×10^6 $\pm 6.46 \times 10^5$	6.65×10^{-3} $\pm 1.71 \times 10^{-4}$	3.50×10^3 $\pm 1.08 \times 10^3$
Dog	$1.16 \times 10^{-5}\dagger$ $\pm 7.50 \times 10^{-7}$	$7.61 \times 10^6\dagger$ $\pm 9.83 \times 10^5$	6.68×10^{-4} $\pm 2.04 \times 10^{-4}$	3.06×10^3 $\pm 1.11 \times 10^3$

* Estimate \pm SD.

$\dagger P < 0.05$ when compared with human serum.

ALBUMIN, TOTAL PROTEIN, AND
LACTIC ACID ANALYSIS

The total protein and albumin concentration of all sera were determined on a Hitachi Model 705® by the Department of Clinical Chemistry of Cincinnati Children's Hospital. Lactic acid concentrations were determined using a Dupont aca®-II by the same Department. The coefficient of variation of the assay methods is less than 5% within the clinically encountered range. α_1 -acid glycoprotein concentrations were not measured in the species studied due to lack of an assay method for determination of this protein in any species other than humans.

CALCULATION OF BINDING
CAPACITY AND AFFINITY

The concentration of free (C_u) and the ratio of bound/free (C_b/C_u) were fitted to the following equation by a nonlinear iterative procedure, NONLIN¹⁵:

$$\frac{C_b}{C_u} = \sum_{i=1}^k \frac{n_i P_i K_i}{1 + K_i C_u}$$

where k is the number of binding site classes, $n_i P_i$ is the capacity, and K_i the affinity of each class of binding sites. This equation is consistent with the analysis of Rosenthal¹⁶ and assumes the various classes of binding sites act independently and are in equilibrium with the free drug in the system.

STATISTICAL ANALYSIS

Statistical comparisons of K_i and $n_i P_i$ were based on the 95% confidence intervals of each parameter estimate.

TABLE 2. Concentrations of Albumin, Lactate and Total Protein in Pooled Serum of Various Species

Species	Total Protein*	Albumin*	Lactate*
Human	7.0	4.6	4.9
Sheep	6.8	3.4	11.2
Monkey	6.7	4.0	8.0
Rat	7.3	4.6	10.5
Dog	5.9	3.1	9.8

* One point estimates. Coefficient of variation of the assay methods is less than 5% within the clinically encountered range.

Results

PHYSIOLOGIC pH

All sera exhibited two classes of binding sites (high affinity, low capacity [class 1]; low affinity, high capacity [class 2]). With the exception of rat serum, a significantly ($P < 0.05$) higher affinity constant (K_1) was observed for the class 1 binding site in all species studied when compared with human serum (table 1). All species studied exhibited a significantly ($P < 0.05$) lower capacity ($n_1 P_1$) for the class 1 binding site when compared with human serum.

The binding parameters ($K_2, n_2 P_2$) for the class 2 binding site in all species studied yielded no significant difference. One point estimates of the total protein, albumin and lactic acid concentrations for all pooled sera studied are shown in table 2.

ACIDIC pH

Comparison with Human Serum: Monkey and sheep sera exhibited a significantly ($P < 0.05$) lower $n_1 P_1$ and significantly ($P < 0.05$) higher K_1 for the class 1 binding site when compared with human serum (table 3). Rat

TABLE 3. Serum Binding Affinities and Capacities at an Acidic pH Caused by Lactic Acid*

Species	$n_1 P_1$ (M)	K_1 (M ⁻¹)	$n_2 P_2$ (M)	K_2 (M ⁻¹)
Human	2.14×10^{-5} $\pm 1.44 \times 10^{-6}$	$6.05 \times 10^5 \dagger$ $\pm 5.69 \times 10^4$	1.49×10^{-4} $\pm 4.25 \times 10^{-5}$	5.03×10^3 $\pm 2.16 \times 10^3$
Sheep	$6.06 \times 10^{-6} \ddagger$ $\pm 7.09 \times 10^{-7}$	$2.21 \times 10^6 \dagger \ddagger$ $\pm 4.36 \times 10^5$	2.69×10^{-4} $\pm 5.47 \times 10^{-5}$	3.27×10^3 $\pm 8.77 \times 10^2$
Monkey	$5.38 \times 10^{-6} \ddagger$ $\pm 5.87 \times 10^{-7}$	$3.44 \times 10^6 \ddagger$ $\pm 1.08 \times 10^6$	2.80×10^{-4} $\pm 8.11 \times 10^{-5}$	3.50×10^3 $\pm 1.23 \times 10^3$
Rat	$7.40 \times 10^{-6} \ddagger$ $\pm 1.44 \times 10^{-6}$	$4.51 \times 10^5 \dagger$ $\pm 1.69 \times 10^5$	$6.19 \times 10^{-4} \ddagger$ $\pm 1.40 \times 10^{-4}$	2.06×10^3 $\pm 5.83 \times 10^2$
Dog	1.62×10^{-5} $\pm 2.19 \times 10^{-6}$	$7.00 \times 10^5 \dagger$ $\pm 2.97 \times 10^5$	3.85×10^{-4} $\pm 1.12 \times 10^{-4}$	2.54×10^3 $\pm 9.86 \times 10^2$

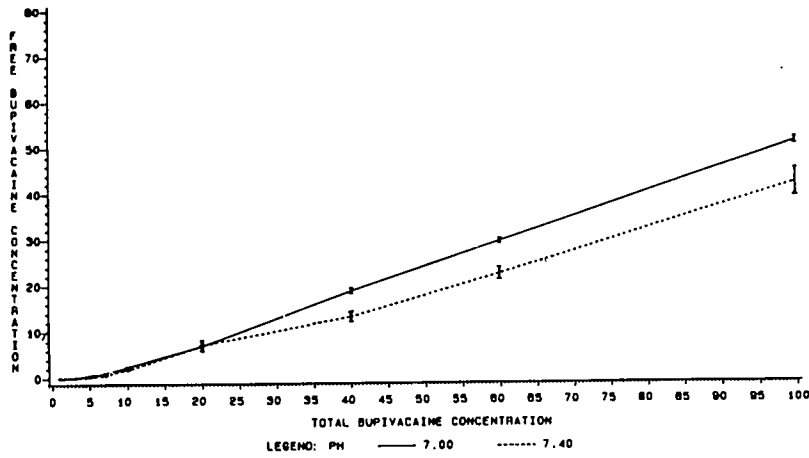
* Estimate \pm SD.

† $P < 0.05$ when compared with physiologic pH in the same species

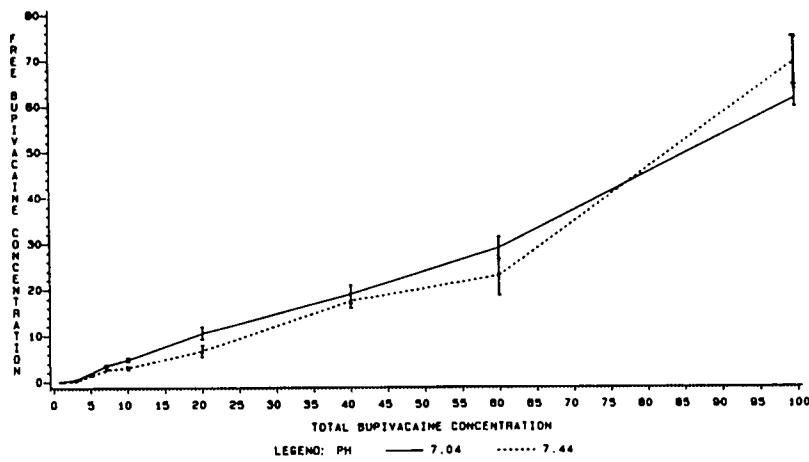
(see Table 1).

‡ $P < 0.05$ when compared with human serum.

A



B



C

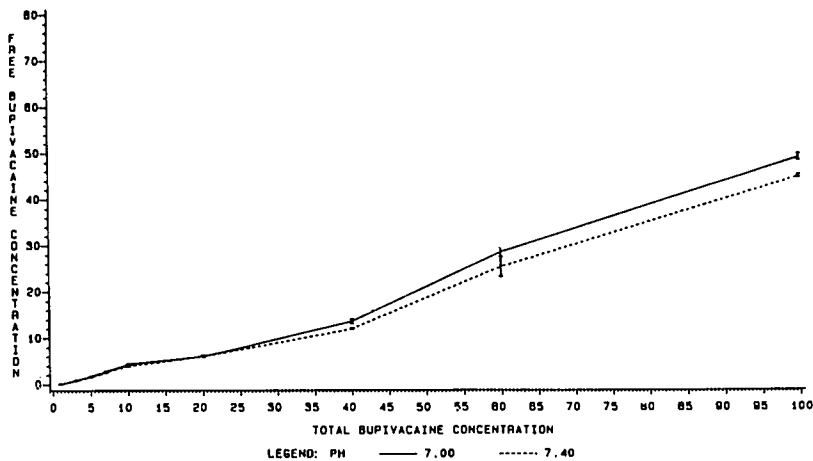


FIG. 1. The influence of lactic acid on the free serum concentration of bupivacaine. A. Human. B. Sheep. C. Monkey. All concentrations expressed as µg/ml (mean ± SD; N = 4).

D

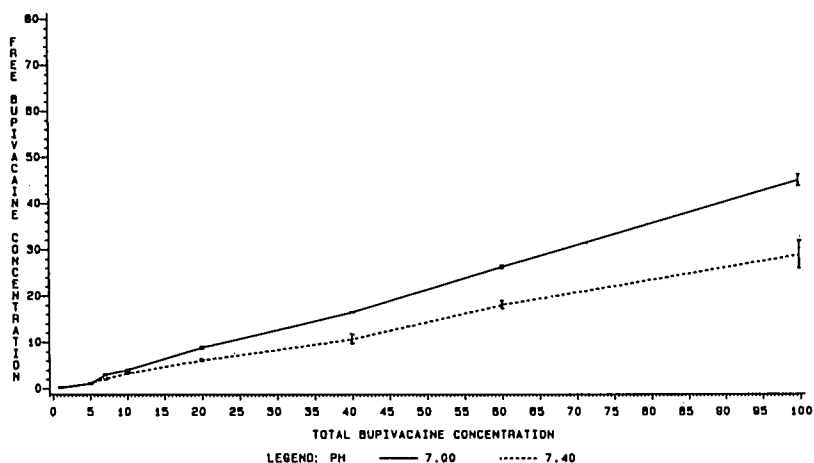
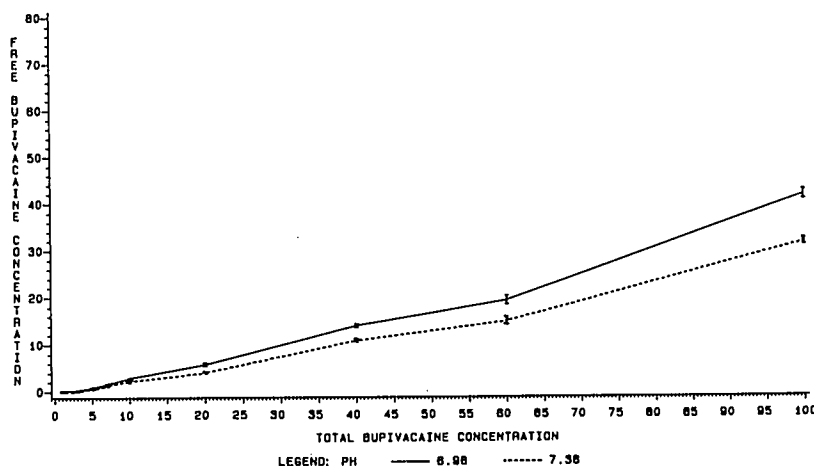


FIG. 1. Continued. The influence of lactic acid on the free serum concentration of bupivacaine. D. Rat. E. Canine. All concentrations expressed as µg/ml (mean ± SD; N = 4).

E



serum displayed a significantly ($P < 0.05$) lower n_1P_1 than humans, whereas dog serum exhibited no significant difference in either parameter of the class 1 binding site.

With exception of the rat, the binding parameters (K_2 , n_2P_2) for the class 2 binding site for the species studied showed no significant difference from human serum. Rat serum exhibited a significantly ($P < 0.05$) higher n_2P_2 .

Effects of Lactic Acid on the Binding Parameters Within a Species: Decreasing the serum pH with lactic acid resulted in a significantly ($P < 0.05$) lower K_1 of the class 1 binding site in all species with the exception of the monkey, which displayed no significant change in this parameter. A significantly ($P < 0.05$) lower n_2P_2 was obtained for the class 2 binding site in the presence of the lactic acid in rat serum. All other binding parameters of the class 2 binding site in the species studied exhibited no significant change in the presence of lactic acid.

Effect of Lactic Acid on the free Concentration of Bupivacaine: The addition of lactic acid to serum resulted in an increase in the free concentration of bupivacaine in all species studied with the exception of the monkey, which displayed no statistical change. Comparative plots of the free bupivacaine concentrations obtained at physiologic and acidic pH versus total bupivacaine serum concentrations are shown in figure 1.

Discussion

Although studies in various animal models have been performed to determine the central nervous system and/or cardiovascular system toxicity of bupivacaine, little or no effort has been taken to determine the disposition of this drug in the various species. It is our contention that the disposition of bupivacaine exhibits interspecies vari-

TABLE 4. Binding Parameters from Individual Dog Serum

Class 1		Class 2	
n_1P_1 (M)	K_1 (M^{-1})	n_2P_2 (M)	K_2 (M^{-1})
$3.94 \times 10^{-10} \dagger \ddagger$	$1.10 \times 10^7 \ddagger$	6.36×10^{-4}	1.58×10^3
$\pm 3.10 \times 10^{-7}$	$\pm 3.80 \times 10^6$	$\pm 1.95 \times 10^{-4}$	$\pm 5.60 \times 10^2$

* Estimate \pm SD (N = 4).

† $P < 0.05$ when compared with pooled dog serum.

‡ $P < 0.05$ when compared with pooled human serum.

ation. Variations in drug disposition between species have been reported for diazepam.¹⁷ Since protein binding is a major determinant of drug disposition, it was chosen as the parameter for this comparison of the various species to humans. The species reported here exhibit some variation from humans that is dependent on the total serum bupivacaine concentration in question. At total bupivacaine concentrations reported to be cardiotoxic in animals (20–60 $\mu\text{g}/\text{mL}$),^{1,2} the sheep and monkey exhibit the closest relationship with humans in terms of free bupivacaine concentration at physiologic pH. Although protein binding is the same within this range, this does not necessarily imply that drug disposition is the same due to other factors affecting disposition (*i.e.*, tissue binding, blood flows, enzyme activity, *etc.*). Data on how other species relate to humans in terms of their disposition of bupivacaine are lacking. It can be seen that investigations into the disposition of the drug in question need to be carried out before data obtained in a particular species can be extrapolated to the human clinical situation.

Another concern of this study was the influence of pH on bupivacaine protein binding among the species. From the results reported here, lactic acid decreases the bupivacaine binding over the entire concentration range studied for all species, with the exception of the monkey. In considering the effect on the binding parameters and the various classes of binding sites, lactic acid appears to exert its influence by decreasing the K_1 of the class 1 binding site in serum (presumed to be α_1 -acid glycoprotein).

Englesson has reported that the degree of convulsive threshold alterations of local anesthetics by respiratory acidosis also is dependent on the underlying metabolic state.¹⁸ A metabolic acidosis has been shown to cause a further decrease in the convulsive threshold of local anesthetics in cats.¹⁸ Munson and Wagman, however, observed that lactic acid does not influence the convulsive threshold of lidocaine in monkeys.⁹ This result may be explained by the observation that monkey serum shows no alteration in bupivacaine protein binding in the presence of lactic acid. Therefore, an increase in free local anesthetic concentration as a result of a decrease in serum pH does not occur in the monkey. This then would result

in no change in the amount of drug available for diffusion into the tissues. In humans, the change in free bupivacaine concentration due to lactic acidosis would result in an increase of 126% or 43% at a total plasma concentration of 1 or 40 $\mu\text{g}/\text{ml}$ respectively. The increase in free bupivacaine would allow more drug (at a particular total blood concentration) to be available for diffusion into the tissues (*i.e.*, heart and brain). This influence could result in an increase in the bupivacaine concentration within the tissues.

The results obtained in this study were acquired using pooled serum from the various species. The individual variations within a species cannot be addressed by this study. Since a larger variation usually is seen in data obtained from individuals, this variation may influence the significant differences observed between the species. However, preliminary results of bupivacaine serum protein binding at normal pH from individual canines (N = 4) fitted simultaneously for estimates of the binding parameters exhibit no significant difference from pooled serum estimates with the exception of the class one binding site capacity (table 4). Further comparison of the individual canine estimates with those obtained from pooled human serum exhibit the same differences between the species as obtained from pooled canine serum. These data do not take into account the individual differences in humans. Therefore, changes in significant differences between the species cannot be addressed totally. Furthermore, the data from individual dogs were obtained and analyzed at a different laboratory than the data on pooled sera. The differences presented here included both interindividual variation and interlaboratory variation.

The differences in the protein binding of bupivacaine between the species may be explained partially by the variation in the concentration of the binding proteins. The current thinking is that for man, α_1 -acid glycoprotein (αAG) accounts for the class 1 site (K_1 , n_1P_1), whereas, albumin accounts for the class 2 site (K_2 , n_2P_2) of the binding classes for local anesthetics.^{19,20} The concentration of the binding proteins determines not only the amount of drug able to be bound but also the remaining free concentration of drug. The differences in αAG concentration, however, cannot be compared because of the lack of an assay method for determination of αAG in any species studied other than humans. Therefore, since bupivacaine protein binding, at any total bupivacaine serum concentration, is dependent on both classes of binding proteins, the differences in the observed bupivacaine protein binding between the species, as a result of the variation in binding protein concentration, cannot be addressed.

Do changes in the binding parameters caused by alterations in protein concentration and lactic acidosis have any clinical significance? It has been reported that disease

states, as well as stress, influence the concentration of both α AG and albumin in humans. These alterations have been found to change the protein binding, disposition, and therapeutic effect of lidocaine.^{8,12,19-21} Increased α AG concentrations also have been reported to have a clinically significant effect on the pharmacokinetics of bupivacaine in cancer patients treated with continuous epidural infusions.²²

Decreases in albumin concentration would have little effect on bupivacaine-free concentration within the clinical range at normal pH. Within the bupivacaine concentration range reported to be toxic in the canine and sheep, a decrease in the albumin concentration would result in an increase in bupivacaine free fraction. Clinically, albumin concentrations as low as 1.5–2.0 g/dl are observed in patients with nephrotic syndrome.²³

The effect of lactic acidosis on bupivacaine binding results in an increase in free bupivacaine concentration. In the range of 1–3 μ g/ml, an average increase of approximately 100% occurs in free concentration of bupivacaine in human serum. In the range reported to be cardiotoxic in sheep and dogs (20–60 μ g/ml), an average increase of approximately 25% occurs (Fig. 1A). The clinical effect of increased free bupivacaine concentration is dependent on the pharmacokinetic parameters of bupivacaine under acidotic conditions. Since these parameters are unknown, the clinical relevance cannot be addressed. However, considering the magnitude of the change occurring, acidosis would exert an influence on protein binding and therefore alter the disposition of bupivacaine.

In summary, bupivacaine serum protein binding is dependent on the total serum binding protein concentration, and for the species studied, this concentration varies from that observed in humans. Lactic acidosis increases the free fraction of bupivacaine over the entire concentration range studied for all species, including humans, with the exception of the monkey, which exhibits no significant change. The results of this study indicate that before the animal studies currently under investigation can be extrapolated to the human clinical situation or compared with each other, further delineation of bupivacaine disposition needs to be investigated in each animal model under both lactic acidosis and physiologic conditions.

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