*Biochemical Pharmacology*, Vol. 34, No. 20, pp. 3779–3781, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00


# Effect of different acidification on *in vitro* human serum bupivacaine binding

(*Received* 30 *November* 1984; *accepted* 3 *May* 1985)

Amide local anesthetics have been reported to bind to various plasma proteins [1–3]. One of the factors which alters local anesthetic protein binding is pH [1, 4–6]. *In vitro* studies of local anesthetic protein binding have used various acidifiers: carbon dioxide [3], hydrochloric acid [4] and lactic acid [6]. Alterations in the *in vitro* protein binding reported with certain of these acidifiers (i.e. hydrochloric or lactic acid) may not be solely dependent on hydrogen ion concentration.

The purpose of this investigation was to compare the effects of various acidifiers (i.e. carbon dioxide, hydrochloric acid and lactic acid) on *in vitro* human serum protein bupivacaine binding.

## *Materials and methods*

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. The sera were pooled, separated into 20 ml aliquots, and stored overnight at 4° before use.

*Ultrafiltration*. Determination of protein binding was performed by using an Amicon Micropartition System (Amicon Corp.; Danvers, MA) equipped with a YMT membrane (Amicon). Preliminary experiments determined that the YMT membrane did not exhibit any nonspecific adsorption of bupivacaine.

Serum protein binding was determined at bupivacaine concentrations of 1.5, 3.5, 6, 17, 36 and 56 μg/ml. Six replications of each concentration were completed for control and for each experimental condition.

The sera, containing various concentrations of bupivacaine, were adjusted to physiologic pH (7.40) by allowing the sera to equilibrate with 5% carbon dioxide/95% air at 37° with mixing for 2 hr. The total concentrations of bupivacaine contained in the sera were determined by gas chromatography at the time of ultrafiltration.

Ultrafiltration was accomplished by subjecting 1 ml of serum to centrifugation at 2050 *g* for 40 min at 30° using a clinical centrifuge (International Equipment Co.; Needham, MA) equipped with a model 803 angle head 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 [7].

The remaining serum from each 20 ml aliquot at each concentration of bupivacaine was then divided into three equal portions and adjusted to a pH of 7.00 using carbon dioxide, hydrochloric acid (Baker Chemical Co.; Phillipsburg, NJ) or lactic acid USP (Baker). The sera were then equilibrated at 37° for 2 hr with mixing and subjected to ultrafiltration as above.

*Analysis*. Bupivacaine concentrations were determined by gas chromatography using a modification of the method of Mather and Tucker [8]. A Hewlett Packard model 5840 gas chromatograph (Hewlett Packard; Avondale, PA) equipped with a 6 ft glass column packed with 3% OV-11 on Chromosorb W AW DMCS (Anspec Co., Ann Arbor, MI) with 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 μg/ml and 12–15% at 10 ng/ml.

*Albumin, $\alpha_1$ acid glycoprotein, total protein, electrolyte, osmolality and lactic acid analysis*. The total protein and albumin concentrations of all sera were determined on a Hitachi model 705 by the Department of Clinical Chemistry of Children's Hospital. Lactic acid concentrations were determined using a Dupont ACA-II. Sodium, potassium, chloride, calcium and total $CO_2$ were determined using a Beckman Astra 8. Osmolality was analyzed using a Precision Systems μOsmette. $\alpha_1$-Acid glycoprotein concentrations were determined by radial immunodiffusion (CalBiochem Behring; LaJolla, CA). All samples were determined in duplicate.

*Calculation of binding capacity and affinity*. The concentration of free ($C_u$) and the ratio of bound/free ($C_b/C_u$) were fit to the following equation by a nonlinear iterative procedure, NONLIN [9].

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

where $k$ is the number of binding site classes, $n_iP_i$ is the capacity and $K_i$ the affinity of each class of binding sites. This equation is consistent with the analysis of Rosenthal [10] and assumes that 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 $n_iP_i$ were based on the 95% confidence intervals of each parameter estimate. All other statistical comparisons were made using a parametric or nonparametric analysis of variance based on the result of the homogeneity of independent variances. These were then followed by a Tukey critical value test (parametric) or a Kruskal–Wallis critical value test (non-parametric). A $P < 0.05$ was considered the minimal level of significance.

## *Results and discussion*

The pooled human serum, irrespective of experimental condition, exhibited no significant difference in the concentrations of total protein (8.1 ± 0.1 g/dl), $\alpha_1$-acid glycoprotein (95.4 ± 2.2 mg/dl), albumin (5.0 ± 0.1 g/dl), sodium (144 ± 0.7 m-equiv./l), potassium (4.3 ± 0.1 m-equiv./l), or calcium (5.1 ± 0.1 m-equiv./l). The addition of the different acidifiers caused significant ($P < 0.05$) alterations in the concentrations of chloride, lactate, and total $CO_2$, and in osmolality, which were dependent on the treatment group (Table 1).

All binding profiles were best described by two classes of binding sites: high affinity, low capacity (Class 1); and low affinity, high capacity (Class 2). A significant ($P < 0.05$) reduction in the affinity ($K_1$) was observed for the Class I binding site for all acidifiers when compared to normal pH serum (Table 2). With the exception of lactic acid, no significant difference in the Class 1 binding capacity ($n_1P_1$) was observed. Lactic acid resulted in a significant ($P < 0.05$) increase in the binding capacity of the Class 1 site.

A significantly ($P < 0.05$) lower affinity ($K_2$) and no significant change in the binding capacity ($n_2P_2$) of the Class 2 site were obtained with lactic or hydrochloric acid. Carbon dioxide, however, caused no change in the affinity of the Class 2 site but did decrease significantly ($P < 0.05$) its binding capacity (Table 2).

Table 1. Effects of different acidifiers on the chloride, lactate and total carbon dioxide concentrations of human serum

| | pH | Chloride (m-equiv./l) | Lactate (m-equiv./l) | Total $CO_2$ (m-equiv./l) | Osmolality (mOsm/l) |
|---|---|---|---|---|---|
| Normal | 7.40 | 105 ± 1* | 3.5 ± 0.1† | 24.2 ± 0.1* | 294 ± 3*† |
| $CO_2$ | 7.00 | 105 ± 1* | 3.5 ± 0.1† | 27.9 ± 0.3*† | 298 ± 2*† |
| Lac | 7.00 | 105 ± 1* | 11.5 ± 0.1*‡§ | 17.5 ± 0.1§ | 311 ± 3‡§ |
| HCl | 7.00 | 113 ± 2*†‡§ | 3.5 ± 0.1† | 18.4 ± 0.1‡§ | 309 ± 3‡§ |

Values are mean ± S.D.
* Significantly different ($P < 0.05$) from HCl.
† Significantly different ($P < 0.05$) from Lac.
‡ Significantly different ($P < 0.05$) from normal.
§ Significantly different ($P < 0.05$) from $CO_2$.

Table 2. Effects of different acidifiers on the protein binding parameters of bupivacaine to human serum

| | pH | $n_1P_1$ | $K_1$ | $n_2P_2$ | $K_2$ |
|---|---|---|---|---|---|
| | | | (estimate ± S.D.) | | |
| Normal | 7.40 | $1.11 \times 10^{-5}$ | $7.64 \times 10^{6}$ | $2.07 \times 10^{-4}$ | $1.86 \times 10^{4}$ |
| | | $\pm 6.22 \times 10^{-7}$ | $\pm 8.77 \times 10^{5}$ | $\pm 1.86 \times 10^{-5}$ | $\pm 2.71 \times 10^{3}$ |
| $CO_2$ | 7.00 | $1.18 \times 10^{-5}$ | $4.27 \times 10^{6*}$ | $1.32 \times 10^{-4*}$ | $1.89 \times 10^{4}$ |
| | | $\pm 8.08 \times 10^{-7}$ | $\pm 5.97 \times 10^{5}$ | $\pm 1.23 \times 10^{-5}$ | $\pm 3.43 \times 10^{3}$ |
| Lac | 7.00 | $1.60 \times 10^{-5*}$ | $3.21 \times 10^{6*}$ | $2.91 \times 10^{-4}$ | $7.51 \times 10^{3*}$ |
| | | $\pm 8.22 \times 10^{-7}$ | $\pm 3.23 \times 10^{5}$ | $\pm 3.93 \times 10^{-5}$ | $\pm 1.60 \times 10^{3}$ |
| HCl | 7.00 | $1.44 \times 10^{-5}$ | $3.82 \times 10^{6*}$ | $3.03 \times 10^{-4}$ | $8.08 \times 10^{3*}$ |
| | | $\pm 1.16 \times 10^{-6}$ | $\pm 5.98 \times 10^{5}$ | $\pm 4.33 \times 10^{-5}$ | $\pm 1.79 \times 10^{3}$ |

* Significantly different from normal pH serum ($P < 0.05$).

Previous studies have reported that decreasing the serum or plasma pH results in a decrease in the local anesthetic protein binding [3, 4–6]. It has been assumed that the decrease in the *in vitro* protein binding of local anesthetics is dependent on the hydrogen ion concentration and, therefore, independent of the acidifier used. We contend that certain acidifiers (i.e. lactic or hydrochloric acid) may not exert their *in vitro* effect based solely on the decrease in pH. The addition of these agents *in vitro* does increase the ionic concentration of the serum and either enhances or reduces the pH effect on *in vitro* bupivacaine protein binding.

A possible explanation of the alteration in the binding parameters of the two classes of bupivacaine binding sites in terms of the acidifier used may be accounted for by shifts in protein conformation. The Class 1 binding site has been reported previously to be present on $\alpha_1$-acid glycoprotein (AAG) [6]. AAG has been reported to exhibit a decrease in its binding affinity for progesterone with any condition (i.e. temperature, pH, solvent environment, etc.) that favors destabilization of AAG conformation [11]. The decrease in affinity of the Class 1 binding site seen with all acidifiers is consistent with the destabilization of the AAG conformational structure.

Changes in ionic concentration or pH have been reported to have little effect on the binding capacity of the binding site for progesterone [11]. This result is consistent with the result obtained with carbon dioxide but does not explain the increases obtained with hydrochloric or lactic acid. The binding capacity exhibited a significant increase with lactic acid and a nonsignificant increase with hydrochloric acid, possibly due to the large error on the hydrochloric acid parameter estimates. From these alterations it would appear that the increase in $Cl^-$ or $Lac^-$ would result in a further destabilizing effect on the AAG conformation. This destabilization would allow more of the AAG to bind bupivacaine.

The Class 2 binding site has been reported to be located on human serum albumin (HSA) [8]. HSA has been reported to show a stabilization of its protein conformation with a decrease in pH [12]. If one assumes that carbon dioxide only results in a pH effect, then the stabilization of HSA conformation does not result in an alteration in the binding affinity of the bupivacaine Class 2 site.

The binding capacity of the Class 2 site was decreased, however, with carbon dioxide. This may be a result of the stabilization of the HSA conformation (tightening of the structure) which reduces the amount of bupivacaine bound. In the case of hydrochloric or lactic acid, a decrease in the binding affinity resulted. Chloride ion has been reported to bind the HSA [13]. This could be a result of an interaction of the $Cl^-$ and possibly that of the $Lac^-$ with bupivacaine. This interaction could be either a competitive or an allosteric inhibition. This explanation would be consistent with no change in the binding capacity.

Compared with normal pH serum (Table 3), carbon dioxide resulted in a significant ($P < 0.05$) increase in the free bupivacaine concentration over the entire concentration range studied. Carbon dioxide also resulted in a significantly ($P < 0.05$) higher free bupivacaine concentration when compared with either lactic or hydrochloric acid (with the exception of hydrochloric acid at a total bupivacaine concentration of 17 μg/ml).

Lactic acid resulted in a significant ($P < 0.05$) increase in the free bupivacaine concentration, compared with control, at all total concentrations studied (with the exception of 6 μg/ml). Lactic acid was significantly ($P < 0.05$) different from hydrochloric acid at total bupivacaine concentrations of 3.5 and 17 μg/ml.

Hydrochloric acid resulted in a significant ($P < 0.05$)

Table 3. Effects of different acidifiers on the free concentration of bupivacaine in human serum

| | Free bupivacaine concentration (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Total concentration (μg/ml) | | | | | |
| | 1.5 | 3.5 | 6.0 | 17.0 | 36.0 | 56.0 |
| Normal | 0.032[a]* ± 0.001 | 0.151[a] ± 0.030 | 0.635[a] ± 0.011 | 3.110[a] ± 0.047 | 10.848[a] ± 0.644 | 18.107[a] ± 1.156 |
| $CO_2$ | 0.051[b] ± 0.007 | 0.194[b] ± 0.006 | 0.826[b] ± 0.025 | 4.172[b] ± 0.058 | 15.217[b] ± 1.268 | 24.897[b] ± 1.510 |
| Lac | 0.041[c] ± 0.006 | 0.180[c] ± 0.017 | 0.657[a] ± 0.098 | 3.790[c] ± 0.378 | 13.233[c] ± 0.544 | 22.372[c] ± 1.116 |
| HCl | 0.041[c] ± 0.006 | 0.135[d] ± 0.025 | 0.695[a] ± 0.018 | 5.402[d] ± 0.480 | 11.400[a] ± 1.047 | 21.357[c] ± 0.932 |

Each value is the mean ± S.D.
* Means with the same letter within the same total concentration range are not significantly different ($P < 0.05$).

increase in the free bupivacaine concentration at 1.5, 17 and 56 μg/ml, when compared with control serum. A significant ($P < 0.05$) decrease in the free bupivacaine concentration resulted at a total bupivacaine concentration of 3.5 μg/ml (Table 3).

In this study, the resulting free bupivacaine concentrations for both lactic and hydrochloric acid were significantly lower than those resulting with carbon dioxide. The increase of either of these two ions and/or the ionic concentrations, resulted in an increase or maintenance of the binding capacity for both the Class 1 and 2 binding sites despite the decrease in pH. The alteration in the overall serum binding capacity (net increase) due to the addition of hydrochloric or lactic acid resulted in a reduction in the magnitude of the *in vitro* pH effect. This explanation assumes that the addition of carbon dioxide represents only a pH effect.

In summary, the effect on the *in vitro* human serum bupivacaine binding was not solely dependent on the reduction of the serum pH. The magnitude of the effect was dependent on the acidifier used. Before *in vitro* protein binding data under acidic conditions can be extrapolated to that occurring *in vivo*, the effect of the acidifier used must be considered.

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*Biochemical Pharmacology*, Vol. 34, No. 20, pp. 3781–3783, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 +0.00


# Effects of γ-glutamyltranspeptidase inhibitor and reduced glutathione on renal acetaldehyde levels in rats

(*Received* 25 *February* 1985; *accepted* 11 *June* 1985)

Much attention has been directed to decreases in tissue-reduced glutatione (GSH) contents following ethanol (EtOH) administration [1–5]. This effect has been discussed in relation to a suppression of GSH synthesis [3] or to an accelerated utilization of GSH detoxifying lipid peroxides [1] or acetaldehyde (AcH), the first metabolite of EtOH [6]. Although Speisky *et al.* [5] concluded that the interaction between GSH and AcH is less important in the liver, the kidney possessing an extremely high γ-glutamyltranspeptidase (γ-GTP) activity [7] may relate to other events since both cysteinylglycine produced from GSH by γ-GTP action and further degradation product, cysteine, have high reactivities to complexes with AcH, non-enzymatically [8]. With a specific γ-GTP inhibitor, L-γ-glutamyl-(*O*-carboxy)-phenylhydrazide (L-OC) [9, 10], and exogenously administered GSH, the present *in vivo* study was carried out to elucidate if the non-enzymatic conjugation reaction between AcH and GSH degradation product(s) has a significant role in AcH and GSH metabolisms after EtOH intake.

## *Materials and methods*

L-OC was obtained through the courtesy of Dr S. Minato, from Fermentation Research Laboratories, Sankyo Co.