Kinetics of Local Anesthetic Esters and the Effects of Adjuvant Drugs on 2-Chloroprocaine Hydrolysis

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A rapid, reliable method for the determination of 2-chloroprocaine in serum was developed. The method, using double-beam ultraviolet spectroscopy, provides rapid, accurate analysis of 2-chloroprocaine in the range of 5.5 to 111 μg/mL (1.5–39 μg/mL), as documented by comparison with the accepted gas chromatographic procedure. The contribution of 4-amino-2-chlorobenzoic acid, the principal metabolite of 2-chloroprocaine, to the total absorbance at 300 nm was examined and found to be negligible. Using the ultraviolet spectrophotometric method, values of the Michaelis-Menten constant (Km) and maximal reaction velocity (Vmax) for hydrolysis of procaine and 2-chloroprocaine by homoyzogous typical, heterozygous, and homozygous atypical plasma cholinesterases were determined. The Km's for the three genotypes were 5.6, 6.2, and 14.7 μM, respectively, for procaine, and 8.2, 17, and 105 μM, respectively, for 2-chloroprocaine. The Vmax's for the three genotypes were similar for all esters. Vmax for procaine was 18.6 ± 0.9 nmol/min/mL serum, while Vmax for 2-chloroprocaine was 58.4 ± 2.1 nmol/min/mL serum. At high concentrations, 2-chloroprocaine acts as an inhibitor of its hydrolysis. The inhibitory effects of lidocaine, bupivacaine, neostigmine, and succinylcholine on 2-chloroprocaine hydrolysis for homzygous typical and atypical variants, respectively, were studied. Competitive inhibition was demonstrated for all four drugs. However, at clinically significant concentrations, only neostigmine and bupivacaine produced high degrees of inhibition. The competitive inhibition constants (Ki) for the typical and atypical variants, respectively, were 3.3 ± 0.3 μM and 15.1 ± 4.8 μM for neostigmine, and 4.2 ± 0.3 μM and 36.9 ± 9.8 μM for bupivacaine. (Key words: Anesthetics, local; bupivacaine; chloroprocaine; lidocaine; procaine. Antagonists, neuromuscular relaxants: neostigmine. Enzymes: pseudocholinesterase. Neuromuscular relaxants: succinylcholine. Measurement techniques: gas–liquid chromatography; ultraviolet spectroscopy.)

The use of local anesthetic mixtures has been reported to produce regional anesthesia with rapid onset and prolonged duration.1,2 Local anesthetic mixtures composed of an amide and an ester are most often recommended to achieve this effect.3,4 Frequently, intraoperative events may necessitate the use of general anesthetics and adjuvant drugs, in addition to the local anesthetics already administered.

Ester local anesthetics are hydrolyzed primarily in plasma, and it is possible that other circulating drugs may interfere with their hydrolysis. Since rapid metabolism is critical to ensure detoxification of ester local anesthetics, a significant inhibition of hydrolysis may increase the risk of adverse reactions. Patients who have atypical plasma cholinesterases may be at even greater risk, since they are known to hydrolyze ester drugs such as succinylcholine more slowly than the normal variant.5

This study was undertaken to determine the magnitudes of the above-mentioned effects on the hydrolysis of 2-chloroprocaine. The hydrolysis kinetics for procaine and 2-chloroprocaine by typical and atypical plasma cholinesterases and the effects of lidocaine, bupivacaine, neostigmine, and succinylcholine on the hydrolysis of 2-chloroprocaine are reported.

Materials and Methods

The following chemicals were used: 2-chloroprocaine hydrochloride (Nesacaine®, Pennwalt); procaine hydrochloride (Novocaine®, Winthrop); lidocaine hydrochloride (Xylocaine®, Astra); bupivacaine hydrochloride (Marcaine®, Breon); dibucaine hydrochloride (Nupercaine®, CIBA), neostigmine methyl sulfate (Prostigmin®, Roche); 4-aminobenzoic acid (Sigma); 4-amino-2-chlorobenzoic acid (Sigma); benzoylcholine chloride (Sigma); succinylcholine chloride (Sigma).

UV Spectrophotometry Method

All measurements were accomplished using a Beckman Model 25 spectrophotometer with the slit width on the three times normal setting. This was necessary to ensure sharp, narrow absorbance peaks for both 2-chloroprocaine and its principal metabolite, 4-amino-2-chlorobenzoic acid, at all concentrations studied. Extinction coefficients (ε) and absorption maxima (λmax) were determined for both 2-chloroprocaine and 4-amino-2-chlorobenzoic acid in 10 per cent serum, 90 per cent serum, and physiologic saline solution (0.9 per cent). The sample cell was 50 μg neostigmine methyl sulfate; the desired concentration of 2-
chlorprocaine and/or 4-amino-2-chlorobenzoic acid, and enough saline solution to bring the volume to 1.0 ml. The reference cuvette contained the same components with the exception of substrate. Similarly, the sample cuvette for measurements in 90 per cent serum contained 0.9 ml serum, 70 μg neostigmine methyl sulfate, the desired concentration of 2-chlorprocaine and/or 4-amino-2-chlorobenzoic acid, and enough Sorensen’s phosphate buffer, pH 7.4, to bring the volume to 1.0 ml. Again, the reference cuvette was prepared in identical fashion, with the omission of substrate. Serum samples also contained neostigmine methyl sulfate at a concentration of 70 μg/ml to inhibit the pseudocholinesterase hydrolysis of 2-chlorprocaine. Stock solutions of 2-chlorprocaine and 4-amino-2-chlorobenzoic acid were prepared just prior to use. Standard curves for 2-chlorprocaine and 4-amino-2-chlorobenzoic acid were obtained by varying the concentration of 2-chlorprocaine from 1 to 30 μg/ml (3.7 to 111 μM) and that of 4-amino-2-chlorobenzoic acid from 0.6 to 16.8 μg/ml (3.3 to 97.5 μM) and measuring the absorbance at 300 nanometers (A300).

**Table 1.** Molar Extinction Coefficients for 2-Chlorprocaine and 4-Amino-2-chlorobenzoic Acid at 300 Nanometers

<table>
<thead>
<tr>
<th></th>
<th>0.9 Per Cent Saline Solution</th>
<th>10 Per Cent Serum</th>
<th>50 Per Cent Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Chlorprocaine</td>
<td>12,900</td>
<td>11,100</td>
<td>6,300</td>
</tr>
<tr>
<td>4-Amino-2-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chlorobenzoic acid</td>
<td>3,200</td>
<td>2,300</td>
<td>500</td>
</tr>
</tbody>
</table>

**FIG. 1.** The contributions of 4-amino-2-chlorobenzoic acid and 2-chlorprocaine to the total absorbance at 300 nm in 10 per cent serum. The contribution of 4-amino-2-chlorobenzoic acid is small. All plots are linear, r = 0.9995.

**GAS CHROMATOGRAPHIC METHOD**

The gas chromatographic procedure used was a modification of the 2-chlorprocaine assay reported by Smith et al.6 2-Chlorprocaine was extracted by placing 1 ml of serum, to which 70 μg/ml of neostigmine methyl sulfate had been added, in a 15-ml screw-topped conical tube. Lidocaine, 2 μg, was added as an internal standard. Ether, 5 ml, and 100 μl of 1 N NaOH were added, and the contents of the tube were mixed on a vortex mixer for 30 sec. The ether layer was aspirated and placed in a clean, 15-ml screw-topped conical tube, and 400 μl of 1 N HCl were added. The contents of the tube were again mixed on the vortex mixer for 30 sec. The ether layer was discarded, and 2 ml of fresh ether and 500 μl of 1 N NaOH were added and mixed on the vortex mixer for 30 sec. The ether was removed and placed in a 5-ml round-bottomed tube, and evaporated to dryness. The residue was dissolved in 50 μl of analytic-grade chloroform, and 2 μl of the resulting solution were injected into a Hewlett Packard Model 5700A gas chromatograph, equipped with a flame ionization detector and a 5 per cent OV-17 on a Chromosorb Q, 300-mesh, 6-foot glass column. Temperature was programmed from 220 C to 250 C at 8 degrees/min.
**Fig. 2.** The contributions of 4-amino-2-chlorobenzoic acid and 2-chloroprocaine to the total absorbance at 300 nm in 90 per cent serum. The contribution of 4-amino-2-chlorobenzoic acid is negligible. All plots are linear, \( r \approx 0.995 \).

Unknown concentrations were determined from a standard curve, which was constructed by varying concentrations of 2-chloroprocaine from 1 to 30 \( \mu \text{g/ml} \) (3.7–111 \( \mu \text{M} \)) and plotting the ratio of the 2-chloroprocaine:lidocaine peak areas obtained from a Spectra Physics Model 4100 computing integrator versus concentration in \( \mu \text{g/ml} \). A constant 2 \( \mu \text{g/ml} \) lidocaine internal standard was used throughout. Standard curves were checked daily for accuracy.

**Hydrolysis Kinetics**

Venous blood was obtained from 22 patients undergoing surgical procedures and from nine volunteers. The blood was allowed to clot and then centrifuged at 2,400 rpm for 10 min. Serum was carefully decanted and either used immediately or frozen at 0°C for future use. Benzoylcholine, dibucaine, and succinylcholine numbers were determined for each sample by methods described previously. Based on these data, subjects were classified as having typical, heterozygous, or atypical plasma cholinesterases. All procaine and 2-chloroprocaine measurements were by the ultraviolet method described above.

Stock solutions of 2-chloroprocaine hydrochloride (325 \( \mu \text{M} \)) and procaine hydrochloride (367 \( \mu \text{M} \)) were prepared daily for use in the hydrolysis studies. The sample cuvette contained 0.10 ml serum; 0.80 ml Sorensen's phosphate buffer, pH 7.40; one of various concentrations of either 2-chloroprocaine (3.7–37 \( \mu \text{M} \)) or procaine (4.2–42 \( \mu \text{M} \)), and enough saline solution to bring the final volume to 1.0 ml. The reference cuvette was prepared identically, except that saline solution was substituted for substrate. Hydrolysis rates for procaine and 2-chloroprocaine in the presence of typical, heterozygous, and atypical plasma cholinesterases were derived by following the disappearance of substrate as a function of time. Inhibition studies were carried out as described above, with the exception that the desired concentration of inhibitor was added to both the sample and reference cuvette. The inhibitor concentrations studied were: lidocaine, 111 \( \mu \text{M} \); bupivacaine, 30.8 \( \mu \text{M} \); neostigmine, 6.0 \( \mu \text{M} \); succinylcholine, 1,000 \( \mu \text{M} \).

Values of the Michaelis-Menton constant \( (K_m) \) and maximal reaction velocity \( (V_{\text{max}}) \) for three volunteers of each genotype were derived from double reciprocal plots according to the method of Lineweaver and Burke. Similarly, competitive inhibition constants \( (K_i) \) for lidocaine, bupivacaine, neostigmine, and succinylcholine were calculated using the following equation:

\[
K_i = \frac{K_{\text{mapp}}}{K_m} - 1
\]

where \( K_i \) is the dissociation constant of the enzyme inhibitor complex; \( I \) is the inhibitor concentration; \( K_{\text{mapp}} \) is the apparent \( K_m \) with inhibitor present; and \( K_m \) is the dissociation constant for the enzyme-substrate complex. Each \( K_i \) was determined using sera obtained from three volunteers with the typical variant and three volunteers with the atypical variant.

All Lineweaver-Burke plots were examined for linearity using standard least-squares regression.
analysis. Normal hydrolysis rates, succinylcholine numbers, Kalow's numbers, dibucaine numbers, and hydrolysis rates in the presence of inhibitors for typical heterozygous and atypical sera were subjected to statistical comparison employing a Student's two-tailed \( t \) test for unpaired data. \( P < 0.05 \) was considered significant.

**Results**

Table 1 gives the extinction coefficients for 2-chloroprocaine and 4-amino-2-chlorobenzoic acid in saline solution, 10 per cent serum, and 90 per cent serum. These were constant over the concentration range studied, demonstrating adherence to Beer's law.

Figures 1 and 2 illustrate the relationships of absorbance and concentration for both 2-chloroprocaine and 4-amino-2-chlorobenzoic acid in 10 per cent and 90 per cent serum, respectively. All curves are linear over the clinically useful range, with correlation coefficients of at least 0.997. It is apparent from these figures that the contribution of 4-amino-2-chlorobenzoic acid to the total absorbance at 300 nm is small, even at extremely high concentrations in 90 per cent serum. The calibration curve for 2-chloroprocaine in the presence of 4-amino-2-chlorobenzoic acid was derived by subtracting the \( A_{300} \) line for 4-amino-2-chlorobenzoic acid from the \( A_{300} \) line for 2-chloroprocaine. The resulting line was used for the determination of hydrolysis rates. The \( A_{300} \) versus concentration curve for 2-chloroprocaine was used for the determination of serum unknowns, since the concentration of 4-amino-2-chlorobenzoic acid was zero.

Figure 3 illustrates the comparison between values determined by ultraviolet spectroscopy and gas chromatography. This figure suggests that the two methods are equally reliable in the range 5.5 to 111 \( \mu \text{M} \) (1.5 to 30 \( \mu \text{g/ml} \)), which is the clinically significant range. The correlation coefficient for these data is 0.994.

Benzoylcholine hydrolysis, dibucaine numbers, and succinylcholine numbers for serum samples were categorized as typical, heterozygous, and atypical plasma cholinesterases (table 2).

Double reciprocal plots were linear for both procaine and 2-chloroprocaine over the initial range of substrate concentrations studied, with correlation coefficients of at least 0.997 (figs. 4 and 5). The \( K_m \) for procaine hydrolysis was 5 \( \mu \text{M} \) in the case of the typical pseudocholinesterase genotype. The \( K_m \) for the heterozygous variant was 1.3 times typical and the \( K_m \) for the atypical variant was 30 times typical. The \( V_{max} \) was the same for all variants. The \( K_m \) and \( V_{max} \) for procaine were identical to those reported by Kalow.11 The \( K_m \) values (mean \( \pm \) SEM) for 2-chloroprocaine hydrolysis were 8.2 \( \pm \) 0.4, 17 \( \pm \) 0.2, and 103 \( \pm \) 31 for typical, heterozygous, and atypical genotypes (n = 5 each), respectively. The heterozygous and atypical samples were significantly different from typical samples (\( P < 0.01 \) and \( P < 0.05 \), respectively). The \( V_{max} \) values were similar for all genotypes. Typical and heterozygous plasma cholinesterases had similar \( K_m \) values, while the \( K_m \) values for the atypical enzyme were significantly greater, and showed greater variation.

Hydrolysis rates for 2-chloroprocaine at a fixed, high substrate concentration were significantly less than predicted by the enzyme kinetic parameters, suggesting substrate inhibition (table 3). Figure 6 illustrates a representative double reciprocal plot over a wide range of substrate concentrations. The hyper-

**Table 2. Quantitative and Qualitative Values of Plasma (Mean \( \pm \) SEM)**

<table>
<thead>
<tr>
<th></th>
<th>Typical Serum (n = 10)</th>
<th>Heterozygous Serum (n = 5)</th>
<th>Atypical Serum (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoylcholine hydrolysis Kalow's # (( \Delta OD/\text{hour/ml} ))</td>
<td>117 ( \pm ) 6.68</td>
<td>117 ( \pm ) 5.68</td>
<td>37.9 ( \pm ) 5.96*†</td>
</tr>
<tr>
<td>Succinylcholine number (per cent I)</td>
<td>76.1 ( \pm ) 9.11</td>
<td>65.1 ( \pm ) 3.85*</td>
<td>15.8 ( \pm ) 4.11†</td>
</tr>
<tr>
<td>Dibucaine number (per cent I)</td>
<td>84.5 ( \pm ) 0.89</td>
<td>66.2 ( \pm ) 2.98*</td>
<td>19.7 ( \pm ) 2.64†</td>
</tr>
</tbody>
</table>

* \( P < 0.001 \) versus typical serum.
† \( P < 0.001 \) versus heterozygous serum.
Fig. 4. $K_m$ and $V_{max}$ values for procaine hydrolysis in a single sample by typical serum, heterozygous serum, and atypical plasma cholinesterase. $K_m$ for typical plasma cholinesterase, 5.0 μM (1.36 μg/ml); for heterozygous serum, 6.2 μM (1.7 μg/ml); for atypical serum, 147 μM (40 μg/ml). The $V_{max}$ for each of the three variants was 10.3 nmol/min/ml serum.

The bolic shape of the curve is typical of classic substrate inhibition. Further verification of this finding was obtained from standard velocity ($v$) versus substrate ($s$) plots. These plots showed an initial increase, followed by a leveling off and, finally, a marked decrease in velocity as a function of continuing increases in substrate concentration. This is the classic pattern for substrate inhibition.

Neostigmine, lidocaine, bupivacaine, and succinylcholine all demonstrate competitive inhibition of

Fig. 5. $K_m$ and $V_{max}$ values for 2-chloroprocaine hydrolysis in a single sample by typical serum, heterozygous serum, and atypical plasma cholinesterase. $K_m$ for typical plasma cholinesterase, 8.2 μM (2.5 μg/ml); for heterozygous serum, 17.0 μM (5.2 μg/ml); for atypical serum, 103 μM (31.6 μg/ml). The $V_{max}$ for each of the three variants was 98 nmol/min/ml serum.
2-chloroprocaine hydrolysis for both typical and atypical genotypes (figs. 7 and 8). At clinically relevant concentrations, only neostigmine and bupivacaine significantly inhibit 2-chloroprocaine hydrolysis. However, this is the case for typical plasma cholinesterase only (table 4).

Discussion

The determination of genotypes is based on the work of Kalow. The values for benzoylcholine hydrolysis, dibucaine number, and succinylcholine number are consistent with the assigned genotypes.

| Table 3. Procaine, 2-Chloroprocaine, and Benzoylcholine Hydrolysis by Typical, Heterozygous, Atypical Plasma Cholinesterases (Mean ± SEM) |
|---|---|---|
| Type | Typical Serum (n = 15) | Heterozygous Serum (n = 5) | Atypical Serum (n = 5) |
| Procaine (110 μM) hydrolysis | 8.29 ± 0.44 | 6.74 ± 0.19* | 2.1 ± 0.40†† |
| 2-Chloroprocaine (98 μM) hydrolysis | 60.2 ± 3.65 | 38.6 ± 1.47† | 12.4 ± 2.5‡‡ |
| Benzoylcholine (200 μM) hydrolysis | 291 ± 16.7 | 291 ± 13.7 | 94.0 ± 14.8†† |

* P < 0.05 compared with typical serum.
† P < 0.001 compared with typical serum.
‡ P < 0.001 compared with heterozygous serum.

Kalow, Kisch et al., and Ting et al. reported K_m for procaine hydrolysis of 4–6 μM. The 5-μM value reported here is in excellent agreement with their findings. The finding that K_m for the atypical enzyme was 30 times higher than that for the typical enzyme is consistent with previous studies of benzoylcholine hydrolysis. Kalow and Hersh et al. reported that the functional difference between typical and atypical enzyme was in affinity (K_m) rather than in catalytic rate (V_max). The data for 2-chloroprocaine hydrolysis further support this hypothesis. There was no biologically significant difference in V_max among the genotypes studied. The K_m for atypical enzyme was 15 times that for the typical enzyme; the K_m for heterozygous enzyme was two times the K_m for the typical enzyme. The V_max with heterozygous serum was slightly and significantly greater than that found with either typical or atypical serum. However, the biological significance of this finding is probably small, and it may, in fact, be artifactual.

| Table 4. Bupivacaine and Neostigmine Inhibition Constants for 2-Chloroprocaine Hydrolysis (Mean ± SEM) |
|---|---|---|
| Type | Bupivacaine K_i (μM) | Neostigmine K_i (μM) |
| Typical (n = 3) | 4.2 ± 0.3 | 3.3 ± 0.3* |
| Atypical (n = 3) | 50.9 ± 9.6† | 15.1 ± 4.8† |

* P < 0.05 versus bupivacaine.
† P < 0.05 versus typical serum.
Fig. 7. Inhibition of 2-chloroprocaine hydrolysis by four drugs using typical plasma cholinesterase. lidocaine $K_i = 62.8 \ \mu M$ (17 $\mu g/ml$); bupivacaine $K_i = 3.3 \ \mu M$ (1.1 $\mu g/ml$); neostigmine $K_i = 4.2 \ \mu M$ (1.4 $\mu g/ml$); succinyldicholine $K_i = 171 \ \mu M$ (62 $\mu g/ml$).

The substrate inhibition of both typical and atypical plasma cholinesterase was first suggested by Kalow.\textsuperscript{11} He reported that procaine hydrolysis rates decreased at high concentrations of substrate. The results reported here confirm that finding with 2-chloroprocaine. Lineweaver-Burke plots over a broad range of substrate were hyperbolic, which is classic for substrate inhibition. The $K_m$ and $V_{max}$ are usually determined for substrate concentrations ranging from a third to three times the expected $K_m$. Substrate concentrations in this range do not inhibit 2-chloroprocaine hydrolysis to any significant extent. 2-Chloroprocaine concentrations in serum in the clinical situation approximate $K_m$, and thus one would not expect substrate inhibition to be of any clinical significance.

Each of four inhibitors studied demonstrated competitive inhibition. The $V_{max}$ for 2-chloroprocaine hydrolysis was not changed by addition of inhibitor, while the $K_m$ appeared to increase. Only neostigmine and bupivacaine had values for $K_i$ that may be clinically significant. In competitive inhibition, $K_i$, like $K_m$, is the inhibitor concentration that results in 50 per cent inhibition of enzymatic activity. The values reported here for neostigmine and bupivacaine fall within the

Fig. 8. Inhibition of 2-chloroprocaine hydrolysis by four drugs using atypical plasma cholinesterase. lidocaine $K_i = 114 \ \mu M$ (30.9 $\mu g/ml$); bupivacaine $K_i = 37 \ \mu M$ (12 $\mu g/ml$); neostigmine $K_i = 15 \ \mu M$ (5 $\mu g/ml$); succinyldicholine $K_i = 608 \ \mu M$ (218 $\mu g/ml$). $K_m$ 2-chloroprocaine = 50 $\mu M$. 

\textsuperscript{11} Kalow, W. J. Am. Chem. Soc. 1937, 59, 1817.
range of concentrations achieved in clinical practice. However, the clinical effect of inhibition of 2-chloroprocaine hydrolysis would probably not be seen in the prolongation of regional anesthesia. The duration of a conduction block is primarily dependent on a number of factors that relate to redistribution, i.e., lipid solubility, $pK_a$, etc., rather than on metabolic clearance rate. The inhibitory effects of neostigmine and bupivacaine may be important when large doses of 2-chloroprocaine are administered. The effect would be to prolong any toxic reaction by allowing the blood levels to remain high for a longer period than if no inhibitor were present. Thus, these drugs can be used in combination without any deleterious effect on anesthesia, but care should be taken to use only the minimum doses needed.

References