Effects of Different Variables on whole Blood Cholinesterase Analysis in Dogs
Fernando Tecles, Cándido Gutiérrez Panizo, Silvia Martínez Subiela and Jose J. Cerón
J VET Diagn Invest 2002 14: 132
DOI: 10.1177/104063870201400207
The online version of this article can be found at:
http://vdi.sagepub.com/content/14/2/132

Published by:
SAGE
http://www.sagepublications.com
On behalf of:
AAVLD
Official Publication of the American Association of Veterinary Laboratory Diagnosticians, Inc.

Additional services and information for Journal of Veterinary Diagnostic Investigation can be found at:
Email Alerts: http://vdi.sagepub.com/cgi/alerts
Subscriptions: http://vdi.sagepub.com/subscriptions
Reprints: http://vdi.sagepub.com/journalsReprints.nav
Permissions: http://www.sagepub.com/journalsPermissions.nav
Citations: http://vdi.sagepub.com/content/14/2/132.refs.html

>> Version of Record - Mar 1, 2002
What is This?
Effects of different variables on whole blood cholinesterase analysis in dogs

Fernando Tecles, Cándido Gutiérrez Panizo, Silvia Martínez Subiela, José J. Cerón

Abstract. The influence of several variables such as sample and reagent storage, anticoagulants, reaction temperature, pH, and substrate concentration on whole blood cholinesterase determination was studied. Storage of nondiluted whole blood samples at room temperature or under refrigeration (4°C) was adequate for short-term storage (3 days to 2 weeks). However, freezing would be more appropriate for long-term storage (≥1 month), and successive thawing and freezing did not produce any loss of cholinesterase activity. All reagents (2,2'-dithiodipyridine as chromophore and acetylthiocholine and butyrylthiocholine as substrates) were stable for 3 months when frozen. Heparin and ethylenediaminetetraacetic acid were the most suitable anticoagulants for whole blood acetylcholinesterase and butyrylcholinesterase determination, because citrate yielded lower acetylcholinesterase values and fluoride inhibited butyrylcholinesterase. Increases in reaction temperature and pH yielded higher cholinesterase values but also increased nonenzymatic substrate hydrolysis. Higher cholinesterase and nonenzymatic substrate hydrolysis values were obtained at higher substrate concentrations were used.

Organophosphate and carbamate insecticides are used extensively as agricultural pesticides and bio-sanitary parasiticides in domestic animals. The main toxic effect of these compounds is the inhibition of cholinesterase (ChE) enzymes. Therefore, determination of ChE activity is used in clinical practice and environmental biomonitoring studies to assess the nature and extent of exposure of wildlife, domestic animals, and humans to organophosphates and carbamates in agricultural and forestry sprays. Moreover, ChE can be used to detect exposure to other contaminants, such as detergents and some metals.

Two main enzymes with ChE activity exist in blood: acetylcholinesterase (AChE) or true cholinesterase, which is present in erythrocyte membranes, and butyrylcholinesterase (BChE) or pseudocholinesterase, which is present in plasma. Traditionally, erythrocytes or plasma have been employed for blood ChE measurements. However, with the exception of avian specimens for which plasma samples are recommended because avian red blood cells do not have ChE activity,29 use of whole blood samples is preferred for several reasons: it is not necessary to separate erythrocytes and plasma, it avoids the poor precision and sensitivity obtained using erythrocyte samples, the sample volume required is reduced, and hemolysis of the sample will not have any negative effect. Furthermore, whole blood analysis has been recommended as a guideline for international standardization of ChE measurements and for use in biological monitoring for environmental studies.19

The Ellman method is currently the most widely used spectrophotometric technique to measure ChE activity in whole blood and is easily adaptable for automated analyzers or plate readers for rapid processing of large numbers of samples. The principle of this method is measurement of the rate of production of thiocholine as the substrate acetylthiocholine is hydrolyzed by the ChE present in the sample. Thiococholine reacts with the chromophore 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) to produce a yellow ion of 5-thio-2-nitrobenzoic acid, and the rate of color production is measured at 412 nm by a spectrophotometer. A modification based in the use of at least 2 substrates, acetylthiocholine and butyrylthiocholine, in different analyses of the same sample for whole blood ChE analysis in domestic animals has been recently recommended because it will allow monitoring of both acetyl- and butyrylcholinesterase activities and a more accurate detection of exposure to anticholinesterase compounds.24 Additionally, 2,2'-dithiodipyridine (2-PDS) has been suggested as an alternative chromophore to DTNB for whole blood ChE assays because the product of the reaction between 2-PDS and thiocholine, 2-thiopyridone, is measured at 343 nm, which mitigates hemoglobin interference and permits the use of fewer sample dilutions.

There are two sources of interlaboratory variation in enzyme determinations: preanalytical sources of variation, including variability due to patient and sample collection and handling, and analytical sources of variation, including factors related with the analytical...
method. Some analytical sources of variation such as chromophore concentration, diluting agents, and the ideal substrate for whole blood ChE analysis have been previously studied. However, there is still a lack of information on how changes in different factors such as sample storage conditions, anticoagulants, reaction temperature, reagent storage, assay pH, and substrate concentration can influence and modify the results of ChE determination in animal whole blood, specially when 2-PDS is used as the chromophore.

Variability in operating procedures for ChE measurements (i.e., differences in assay pH, substrate concentration or reaction temperature) occurs between different laboratories, even when the same basic analytic method is used.

Inappropriate storage procedures and the lack of standardization have been defined as the main problems that affect ChE determinations and limit the practical application of these data. To further define sources of variation, this study was undertaken to examine the influence of sample storage conditions and diverse technical parameters (anticoagulants, temperature, reagent stability, pH, and substrate concentration) on whole blood ChE assays.

Materials and methods

Sample collection. Heparinized whole blood was obtained from venipuncture of cephalic veins of 10 healthy dogs (German Braco) and mixed to obtain a pool that was used for the different tests. All animals had no history of exposure to any organophosphate or carbamate compound in the last 2 mo. Whole blood was diluted with distilled water (1:50 dilution ratio) before analysis.

Cholinesterase analysis. Cholinesterase activity in whole blood was measured using an automated method in a compact multiparametrical autoanalyzer (Coulter Profile Analyzer). 2-PDS was used as the chromophore at a final concentration of 8 x 10^{-3} M. Phosphate buffer (pH 7.5) was employed to prepare buffer–chromophore solution. Two different substrates were used, acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI), at a final concentration of 1 x 10^{-3} M for both substrates.

Cholinesterase activity was expressed as micromoles of substrate hydrolyzed/ml/min and calculated as follows: final activity = change in absorbance at 340 nm wavelength/min x 1.55 x initial dilution. The conversion factor (1.55) = (1.000/7.060 x 1) x (275/25), where 1.000 = factor for conversion from mmol/ml to μmol/ml; 7.060 M^{-1} cm^{-1} = absorptivity of 2-thiopyridone at 340 nm wavelength; 1 cm = path length; and 275/25 = dilution in the cuvette.

To calculate backgrounds, a blank containing buffer–chromophore and substrate was assayed to assess nonenzymatic substrate hydrolysis for each sample. An additional blank consisting of diluted blood (sample) and buffer–chromophore was also analyzed to calculate the reaction between chromophore and blood thiol groups different from acetyl- or butyrylthiocholine, such as glutathione, as previously recommended. Activities for both blanks were subtracted from total ChE activities of samples.

Stability of ChE. To determine stability of whole blood ChE activity, a pool of diluted and nondiluted heparinized whole blood was separated in aliquots and stored at 3 different temperatures: 25, 4, and -20°C. Samples were analyzed at 1, 3, 7, 14, 30, 90, and 180 days of storage, and results were compared with those obtained on the day of blood collection.

Five whole blood aliquots were thawed at different temperatures (4, 25, and 60°C) and frozen once daily for 5 days. Cholinesterase activity was monitored daily to determine effect of successive thawing and freezing.

Effect of anticoagulants. Whole blood was obtained from venipuncture of cephalic vein of 10 healthy dogs (German Braco). To avoid clotting, aliquots of each sample were mixed immediately after extraction with 4 different anticoagulants: heparin (0.5 mg/ml), ethylenediaminetetraacetic acid (EDTA, 0.2 mg/ml), sodium citrate (0.38 mg/ml), and sodium fluoride (1 mg/ml). Results obtained with each anticoagulant were compared to assess the effect on ChE activity.

Effect of temperature. A pool of heparinized whole blood was separated in 5 aliquots and assayed at 4 different reaction temperatures (25, 30, 37, and 40°C). For these analyses, a COBAS MIRA PLUS analyzer was used because the Coulter Profile Analyzer only allowed assays to be performed at 30 and 37°C. The effects of temperature on nonenzymatic substrate hydrolysis and buffer–chromophore blank were also tested.

Reagent stability. All freshly prepared reagents were separated in aliquots and stored at 3 different temperatures: 25, 4, and -20°C. Reagents were employed after 6 hours and 1, 2, 7, 14, 30, and 90 days of storage for ChE analysis of 5 whole blood aliquots. Results were compared each time with those obtained with reagents prepared freshly. Chromophore stability was compared using fresh acetylthiocholine as substrate. Substrate stability was tested employing fresh 2-PDS as chromophore.

Effect of pH. To assess effect of reaction pH on ChE determination, 5 aliquots from a pool of canine whole blood were analyzed using buffer–chromophore solution prepared at 5 different pHs: 7.0, 7.5, 8.0, 8.5, and 9.0. The chromophore 2-PDS and 2 substrates (ATCI and BTCI) were used. Effect of pH on nonenzymatic substrate hydrolysis and the buffer–chromophore blank was also tested.

Effect of substrate concentration. The 1 M dilutions of ATCI and BTCI substrates were prepared by diluting 0.159 g and 0.1745 g of ATCI and BTCI, respectively, in 5 ml of distilled water. From these 2 initial dilutions, 5.5 x 10^{-2} M, 1.1 x 10^{-2} M, 5.5 x 10^{-3} M, and 1.1 x 10^{-3} M concentrations of each substrate were also made. All dilutions were used for ChE determination of 5 aliquots from a pool of canine whole blood, giving final concentrations in reaction mixtures of 1 x 10^{-2} M, 5 x 10^{-3} M, 1 x 10^{-3} M, 5 x 10^{-4} M, and 1 x 10^{-4} M, respectively. Results obtained with each substrate concentration were compared. Nonenzymatic substrate hydrolysis and the buffer–chromophore blank were tested to assess changes due to substrate concentration.
Figure 1. Canine whole and diluted blood cholinesterase (ChE) stability under different temperature storage conditions: 25, 4, and −20 C. A, ChE activity obtained using acetylthiocholine iodide as substrate. B, ChE activity obtained using butyrylthiocholine iodide as substrate. ChE activity is expressed as μmol of substrate hydrolyzed/ml/min. All results have been corrected for values obtained with blanks.

Figure 2. Canine whole and diluted blood cholinesterase (ChE) stability after successive thawing and freezing. A, ChE activity obtained using acetylthiocholine iodide as substrate. B, ChE activity obtained using butyrylthiocholine iodide as substrate. ChE activity is expressed as μmol of substrate hydrolyzed/ml/min. All results have been corrected for values obtained with blanks.

Table 1. Effect of different anticoagulants on determination of canine whole blood cholinesterase activity (mean ± SD μmol of substrate hydrolyzed/ml/min).

<table>
<thead>
<tr>
<th>Anticoagulant</th>
<th>Heparin†</th>
<th>EDTA</th>
<th>Citrate</th>
<th>Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>AChE</td>
<td>1.70 ± 0.11</td>
<td>1.61 ± 0.07</td>
<td>1.57 ± 0.09</td>
<td>1.74 ± 0.10</td>
</tr>
<tr>
<td>BChE</td>
<td>1.40 ± 0.14</td>
<td>1.37 ± 0.13</td>
<td>1.30 ± 0.14</td>
<td>1.20 ± 0.16</td>
</tr>
</tbody>
</table>

* AChE = acetylcholinesterase; BChE = butyrylcholinesterase.
† Control group for statistical analysis.
Both acetyl- and butyrylthiocholine stored at −20 C were stable for 3 months. At 4 C, acetylthiocholine and butyrylthiocholine were stable for 2 weeks, although significant changes (P < 0.001) were seen after this time (significant differences were not included in the figure). Significant differences (P < 0.001) were seen after 24 hours when acetylthiocholine was stored at room temperature, whereas no significant differences were observed during 2 weeks of butyrylthiocholine storage.

**Effect of pH.** Highest ChE values were obtained when pH was between 8.0 and 8.5, and ChE values decreased at higher pHs (Table 3). However, nonenzymatic hydrolysis of substrates increased significantly (P < 0.001) at these pH values. BTCI was the substrate that showed lower nonenzymatic hydrolysis.

**Effect of substrate concentration.** Changes in ChE activity due to modifications in substrate concentration are presented in Table 4. Cholinesterase activity reached its maximum at 5 × 10⁻³ M ATCI and decreased when a higher substrate concentration was used. However, when BTCI was used, enzymatic activity did not decrease at the highest substrate concentration (1 × 10⁻² M). Nonenzymatic hydrolysis of both substrates showed a significant increase (P < 0.001) when substrate concentrations were higher than 1 × 10⁻³ M. ATCI showed higher nonenzymatic hydrolysis than did BTCI.

**Discussion**

All ChE assays were performed using 2 substrates (acetyl- and butyrylthiocholine) because the use of both substrates is necessary for a complete characterization of ChE activity in canine whole blood, in contrast to other species such as ruminants or swine for which only acetylthiocholine is needed. The presence of AChE and BChE in canine whole blood in a similar proportion to that in humans makes the dog an ideal animal model for ChE studies. 2-PDS was used in all assays because ChE analysis with this chromophore has been strongly recommended for whole blood determination because it allows the use of more concentrated samples, increasing the sensitivity of the assay especially when carbamates are tested.

Storage of nondiluted whole blood samples at room temperature or refrigerated from 3 days to 2 weeks did not result in changes in ChE activity. Thus, these temperatures are adequate for short-term storage. The use of 25 C or 4 C for long-term storage presented 2 main problems for canine blood: 1) nondiluted samples clotted in 2–3 weeks under these storage conditions, preventing analysis; and 2) dilution of the sample produced a significant decrease in ChE activity after only 24 hours. In contrast to humans, where whole blood showed poor enzyme stability, whole and diluted canine blood enzyme activity was stable for 1 month.
Table 3. Effect of pH on determination of canine whole blood cholinesterase activity (mean ± SD μmol of substrate hydrolyzed/ml/min).

<table>
<thead>
<tr>
<th>Activity*</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.0</td>
</tr>
<tr>
<td>AChE</td>
<td>1.84 ± 0.01***</td>
</tr>
<tr>
<td>N.E. ATCI</td>
<td>0.10 ± 0.01***</td>
</tr>
<tr>
<td>BChE</td>
<td>1.48 ± 0.01***</td>
</tr>
<tr>
<td>N.E. BTCI</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Sample–chrom.</td>
<td>0.24 ± 0.01***</td>
</tr>
<tr>
<td>Corrected AChE</td>
<td>1.50 ± 0.01***</td>
</tr>
<tr>
<td>Corrected BChE</td>
<td>1.19 ± 0.01***</td>
</tr>
</tbody>
</table>

* AChE = acetylcholinesterase; BChE = butyrylcholinesterase; N.E. ATCI = nonenzymatic hydrolysis of acetylthiocholine blank; N.E. BTCI = nonenzymatic hydrolysis of butyrylthiocholine blank; Sample–chrom. = sample–chromophore blank. Corrected values were obtained after nonenzymatic hydrolysis and sample–chromophore blanks were subtracted.
† Control group for statistical analysis; *** P < 0.001.

Table 4. Effect of substrate concentration on determination of canine whole blood cholinesterase activity (mean ± SD μmol of substrate hydrolyzed/ml/min).

<table>
<thead>
<tr>
<th>Activity*</th>
<th>Substrate concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × 10^-4 M</td>
</tr>
<tr>
<td>AChE</td>
<td>1.21 ± 0.02***</td>
</tr>
<tr>
<td>N.E. ATCI</td>
<td>0.02 ± 0.01***</td>
</tr>
<tr>
<td>BChE</td>
<td>0.85 ± 0.01***</td>
</tr>
<tr>
<td>N.E. BTCI</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>Sample–chrom.</td>
<td>0.36 ± 0.01</td>
</tr>
<tr>
<td>Corrected AChE</td>
<td>0.83 ± 0.02***</td>
</tr>
<tr>
<td>Corrected BChE</td>
<td>0.48 ± 0.01***</td>
</tr>
</tbody>
</table>

* AChE = acetylcholinesterase; BChE = butyrylcholinesterase; N.E. ATCI = nonenzymatic hydrolysis of acetylthiocholine blank; N.E. BTCI = nonenzymatic hydrolysis of butyrylthiocholine blank; Sample–chrom. = sample–chromophore blank. Corrected values were obtained after nonenzymatic hydrolysis and sample–chromophore blanks were subtracted.
† Control group for statistical analysis; *** P < 0.001.
significant differences will appear in ChE activity results between laboratories that use different measurement temperatures. Many methods have been described using 37°C, a temperature at which most mammalian ChEs display an optimal rate of activity. However, room temperature is frequently used for laboratory measurements, thus avoiding the need for heating systems in the apparatus of measurement, and most commercial kits for ChE determination suggest the use of a reaction temperature of 25°C. Nostrand et al. recommended that assays should be conducted at room temperature rather than at 37°C in cases of suspected carbamate exposure to minimize ChE reactivation.

Conversion factors for transforming results obtained at different temperatures have been calculated for human plasma and canine plasma samples. However, in the present study (Table 5), slight differences in conversion factors were found for canine whole blood samples. These differences should be taken into consideration to obtain a more accurate temperature conversion. In general, the correction factor for temperature should always be derived from the same enzyme source, tissue preparation procedures, and method of analysis to prevent potential errors.

Both ATCI and BTCI were stable for at least 3 months when frozen, which is a longer time than that reported previously for ATCI. No reports have been found concerning 2-PDS storage. In our study, this chromophore was stable for at least 3 months. This characteristic will allow a more simple and economic use of these reagents.

The activity of ChE enzymes is pH dependant; protonation of the active site amino acids is influenced by the pH of the surrounding medium, and the nucleophilicity (capability of nucleophilic attack on substrate) of the enzyme is directly dependant upon the pH at the active site. A significant increase of AChE and BChE activity was observed when higher pHs were used. However, if these values were corrected for nonenzymatic substrate activity, as has been previously suggested, higher ChE activity would be observed with pHs between 8.0 and 8.5. A great variety of pHs, 8.0, 7.9, 7.6, 7.5, 7.4, 7.2, and 7.0, can be found among different laboratories. In 1 study, pH 8.0 was used for sample dilution, but pH 7.0 was used for DTNB buffer because it was more stable. The choice of an ideal pH can be difficult, because higher pHs yield higher ChE activity but increase substrate blank activity, thus reducing assay sensitivity. In the present study, pH 7.5 was used, an intermediate pH that is inside the reference range of canine physiologic blood pH (7.37–7.51).

Regarding substrate concentration, the highest whole blood AChE and BChE activity was obtained when substrate concentrations were 5 × 10^{-3} M and 1 × 10^{-3} M for ATCI and BTCI, respectively. ATCI concentration of 1 × 10^{-2} M caused AChE inhibition, but this effect was not observed for BChE, and this enzyme continued to increase with BTCI concentrations of 1 × 10^{-2} M. Differences between AChE and BChE behavior with excess of substrate have been previously reported in samples other than whole blood. Inhibition of AChE in erythrocytes has been demonstrated in the presence of high concentrations of ATCI, whereas plasma BChE inhibition does not occur when high levels of BTCI are present. However, the low ChE values obtained with substrate concentrations of 1 and 5 × 10^{-4} M could be explained because these concentrations were not sufficiently high to achieve zero order kinetics in the enzymatic reaction. In this work, the pattern of canine AChE and BChE activities using different concentrations of specific substrates was similar to that obtained with human whole blood samples. However, nonenzymatic hydrolysis of both substrates was significantly increased when concentrations exceeded 1 × 10^{-3} M. Based on these results, substrate concentrations of 1 × 10^{-3} M should be used for AChE and BChE whole blood determinations.

Commercial kits recommend the use of substrate concentrations of ≥5 × 10^{-3} M, and in an interlaboratory study 77% of participants used ATCI concentrations >4.68 × 10^{-3} M. At such reagent concentrations in canine samples, an additional measurement and correction for nonenzymatic substrate hydrolysis should be done to prevent erroneous increases of ChE activity. The lack of sample correction for blanks leads to overestimations of ChE activity in organophosphate-inhibited whole blood samples, and overestimations increased the more samples were inhibited and could be >50% of true activity.

Based upon unpublished data, similar recommendations concerning storage conditions, anticoagulants, reaction temperature, pHs, and substrate concentration should be followed when DTNB is used as a chromophore.

Results obtained in this work contribute to a better understanding of the factors that influence ChE determination and to the establishment of technical guide-

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Human plasma ATCI</th>
<th>Human plasma BTCI</th>
<th>Canine whole blood ATCI</th>
<th>Canine whole blood BTCI</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>30°C</td>
<td>0.78</td>
<td>0.85</td>
<td>0.74</td>
<td>0.85</td>
</tr>
<tr>
<td>37°C</td>
<td>0.60</td>
<td>0.70</td>
<td>0.65</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* BTCI = butyrylthiocholine iodide.
† ATCI = acetylthiocholine iodide.

Variables in dog whole blood cholinesterase
lines for ChE analysis that could optimize the methods and reduce the interlaboratory variability of the analytical procedure. Knowledge and control of these sources of variation would improve sensitivity of the assay, which is especially important for accurately detecting subclinical exposures to organophosphate and carbamate compounds, which produce lower degrees of ChE activity depression. There is a physiopathologic justification for worrying about small depressions in activity. Dose/response curves of ChE activity versus organophosphate concentration in vitro are steep, and once started down the inhibition curve, a little bit more pesticide may become much more dangerous than a similar amount given to an unexposed animal.3 This study was focused on canine samples, and further studies are needed to determine whether assay conditions have similar effects on whole blood ChE results in other animals.

**Acknowledgement**

We are grateful to Dr. Carmen Lagares, director of Murcia University Animal Resources Center, for technical support during the experimental trial.

**Sources and manufacturers**

a. Coulter Scientific, Margency, France.

b. Sigma Chemical Co., St. Louis, MO.

c. Analema, Vigo, Spain.

d. Aquisel, Barcelona, Spain.

e. ABX Diagnostics, Montpellier, France.

f. Statistical Graphics Corp, Rockville, MD.

g. University Animal Resources Center, for technical support.

**References**


