A Rapid, Semi-Automated Method for Determining Dibucaine Numbers

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Determination of the dibucaine number of serum permits the differentiation of genetically normal serum cholinesterase from the dibucaine-resistant variant. This communication presents a method for the rapid determination of dibucaine numbers on an automated analyzer that is in widespread use. With this method, dibucaine numbers for seventy-eight normal, four heterozygous, and five dibucaine-resistant sera were 79 ± 3, 60 ± 10, and 20 ± 3 (± SD), respectively. There was good agreement between the orginal method and the automated method with a correlation coefficient of 0.98 on split sample comparison of thirty-eight normal, four heterozygous, and five abnormal sera. Advantages of this method over the original include the lack of need for advance preparation and that it can provide results within one-half hour of phlebotomy. Key words: Complications: apnea. Enzymes: cholinesterase. Measurement techniques: dibucaine number. Neuromuscular relaxants: succinylcholine.

The dibucaine number determination, first reported in 1957,1 has been accepted as the method for differentiating the dibucaine-resistant variant of serum cholinesterase (EC 3.1.1.8) from the normal form.2 The principle of the determination is that the normal enzyme will be inhibited about 80 per cent by dibucaine at 1 \( \times 10^{-5} \) M while an abnormal variant will be inhibited by only about 20 per cent by the same concentration.1 This assay permits one to identify patients at risk for an abnormally prolonged response to succinylcholine and also provides diagnostic information in cases of prolonged apnea following succinylcholine. The population incidence of the dibucaine-resistant variant of serum cholinesterase is about 1:2,500.3 To identify other genetic variants of the enzyme, additional inhibitors of the enzyme have been employed. These include: fluoride,4 chloride, formaldehyde, several alkyl alcohols, and others.5 Of these, only fluoride has proven to be useful clinically, with the incidence of the fluoride-resistant variant being reported to range from 1:154,000 to 1:300,000.3,6

Dibucaine numbers have been reported with assays utilizing several substrates, including benzoylcholine1 (the original method), propionylthiocholine,5,7 acetylthiocholine and butyrylthiocholine,5 and automated assays using these substrates have been described.8 Although these newer methods work, widespread replacement of the original assay has not occurred, in part because of tradition2 and in part because all of the methods, including the original, require special technical skills and are time-consuming.

This paper reports an alternative method which provides dibucaine numbers within one-half hour of phlebotomy, involves minimal technician time, and requires essentially no advance preparation.

Materials and Methods

This project was approved by the Human Studies Committee of the University of Virginia. Blood for serum cholinesterase and dibucaine number determinations was obtained from adult volunteers, from patients with documented sensitivity to succinylcholine, and from relatives of these patients. Serum was separated by centrifugation and was assayed immediately or was stored at -20°C for later assay. Serum cholinesterase activity without and with dibucaine was determined by at least two of the three following methods. First, serum samples were diluted 100x and assayed in a Beckman® 25 recording ultraviolet spectrophotometer at 26°C with benzoylcholine without and with dibucaine (1 \( \times 10^{-3} \) M) using the method of Kalow and Genest.1

Second, serum samples were assayed manually at 37°C with butyrylthiocholine (5.4 \( \times 10^{-4} \) M) using the method of Ellman et al. as modified by Klingman et al.9,10 Some of these assays were done after adding dibucaine, in concentrations from 1 \( \times 10^{-6} \) to 6 \( \times 10^{-4} \) M.

Finally, serum samples without and with dibucaine were assayed for serum cholinesterase activity on a DuPont® Automatic Clinical analyzer (ACA). This instrument is in widespread use, requires no operator intervention except for sample loading and maintenance, and is capable of rapidly performing over 35 different clinical laboratory determinations. Assays are run at 37°C and butyrylthiocholine is used as the substrate for serum cholinesterase. The normal range for serum cholinesterase activity with this method is 7 to 19 interna-
tional units (IU)/ml of serum, and the assay is linear from 0 to 14 IU. The reagent packs for the assay have a shelf life of one year. A detailed description of the method is available from the manufacturer.‡

Whenever dibucaine was required in the automated assay, serum was premixed with crystalline dibucaine HCl to permit the addition of dibucaine without diluting the serum. The desired dibucaine concentration was achieved by adding precisely measured aliquots of serum (Pipetman®, Rainin Inst. Co.) to 12 × 75 mm borosilicate tubes each containing a known amount of dry dibucaine HCl. The tubes had been prepared earlier by adding an aliquot of dibucaine in aqueous solution to each tube and then lyophilizing the solutions to dryness. In all cases, the volume of serum used to reconstitute the lyophilized dibucaine was equal to the volume of the dibucaine solution prior to its lyophilization. The concentration of dibucaine dissolved in the serum was greater than the desired final concentration in the reaction mixture by a factor equal to the dilution of serum by the ACA.§

To determine the concentration of dibucaine needed to differentiate the variants of serum cholinesterase with the automated assay, sera from five patients with genetically normal serum cholinesterase, from five with the dibucaine-resistant variant, and from three proven to be heterozygous using the original method¹ were assayed on the ACA in series without and with dibucaine ranging in concentration from 1 × 10⁻⁵ M to 2 × 10⁻⁴ M in the reaction mixture (fig. 1). A concentration of 1 × 10⁻⁴ M was selected to differentiate the normal enzyme from the dibucaine-resistant variant in further experiments.

Dibucaine numbers for both manual and automated assays were calculated with the following formula:

\[
\text{Dibucaine Number} = \left( 1 - \frac{\text{inhibited activity}}{\text{uninhibited activity}} \right) \times 100
\]

Precision of the automated method within a single run was established by running twenty repetitive assays with two genetically normal sera with low and high activities and with an enzyme verifier (DuPont) assayed directly and after dilution with DuPont® Enzyme Diluent. Similarly, dibucaine numbers of two normal sera, the verifier and diluted verifier were determined once per day for 18–22 days (not necessarily consecutive) over a one-month period to establish a day-to-day precision of the method. Ranges of normal values for the method were determined by assaying sera from 34 males and 34 females with genetically normal serum cholinesterase.

Agreement of the automated assay with the original method¹ was established by comparing dibucaine numbers in 47 sera (including five homozygous and four heterozygous atypical sera) assayed by both methods. The sera assumed to be heterozygous were from parents of patients who had the atypical variant.

Stability of dibucaine following lyophilization was confirmed by two independent methods. First, the amount of dibucaine in representative tubes prepared in lots at intervals spanning one year and stored at room temperature was assayed by gas chromatography (Hewlett-Packard® 5750). Second, dibucaine numbers were determined in six sera using lyophilized dibucaine from the lots that had been chromatographed.

Results

Pilot experiments reported in abstract form¹¹ demonstrated that the substrate butyrylthiocholine could be substituted for benzoylcholine in both manual and automated assays for the determination of dibucaine numbers, and that the results using butyrylthiocholine closely approximated those of the original method. The following data were used to define the characteristics of the automated assay.

Precision of the automated assay in four sera was established both within a single run (table 1) and from day to day (table 2). The range of dibucaine numbers

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‡ ACA Test Methodology, Pseudocholinesterase. DuPont Co. Instrument Products, Automated Clinical Analysis Division, Wilmington, Delaware 19898.
§ Benzoylcholine, butyrylthiocholine and dibucaine HCl were obtained from Sigma Chemical Co. Additional dibucaine HCl was a gift from Dr. William Grovier of Ciba-Geigy. “PCH” packs for the automated assays were provided by Dr. William Naccarato of the DuPont Corporation.
TABLE 1. Precision of the Automated Assay within a Run

<table>
<thead>
<tr>
<th></th>
<th>PchE Activity (±SD) IU/ml</th>
<th>Dibucaine Number (±SD)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dibucaine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1.0 x 10^-5 M</td>
<td></td>
</tr>
<tr>
<td>High PchE activity</td>
<td>19.36 ± 0.16</td>
<td>4.18 ± 0.21</td>
</tr>
<tr>
<td>Enzyme verifier</td>
<td>10.76 ± 0.09</td>
<td>2.71 ± 0.07</td>
</tr>
<tr>
<td>Diluted enzyme</td>
<td></td>
<td></td>
</tr>
<tr>
<td>verifier</td>
<td>5.77 ± 0.04</td>
<td>1.33 ± 0.06</td>
</tr>
<tr>
<td>Low activity</td>
<td>4.15 ± 0.04</td>
<td>1.07 ± 0.03</td>
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in 78 normal sera was 79.0 ± 3.2 (SD) with the extent of inhibition being the same in sera from males and females, 79.0 ± 3.2 and 79.1 ± 2.9, respectively.

Split sample comparisons between the original method and the automated method were in good agreement (fig. 2). The duration of incubation of serum with dibucaine prior to enzyme assay did not affect the extent of inhibition (data not shown).

Dibucaine remained stable and active following lyophilization and storage. The amount of dibucaine determined by gaschromatography in representative tubes from lots prepared at intervals spanning one year was within 15 per cent of the calculated value. In addition, dibucaine numbers determined using tubes that had been stored for up to one year were identical to those determined using freshly prepared tubes.

Attempts to adapt the automated assay for the determination of fluoride numbers were unsuccessful. Although differential inhibition of normal and a fluoride-resistant variant was observed, there was little correlation between the automated method and the method of Harris and Whittaker (data not shown).

**Discussion**

Since the original report of the determination of dibucaine numbers a multitude of alternate methods have been developed, of which this report is one more. The advantages of the method presented here, however, appear to make it an attractive alternative to both the original and later methods. These include the rapidity of the assay (results can be available within one-half hour of venipuncture), the lack of need for advanced preparation other than preparing tubes with dibucaine and routine maintenance of the Automatic Clinical Analyzer. No special training is required beyond that needed to operate the ACA. The original method requires hours of technician time, the use of specialized equipment, and is thus not ideally suited for assaying a single specimen. In contrast, the automated method can provide data quickly from single or multiple specimens in any clinical laboratory that has an ACA. It can be used as a preoperative screen and also as an aid in the differential diagnosis of postoperative apnea, although caution has been advised in interpreting dibucaine numbers determined with sera obtained during prolonged paralysis. Further, the close agreement between this method and the original make interpretation of results unambiguous to those familiar with the test.

The assay conditions with the ACA differ substantially from the original method. The original is carried out at 26°C in phosphate buffer with benzoylcholine while the ACA performs the automated assay in a similar buffer but at 37°C with butyrylthiocholine. Also, the concentrations of dibucaine needed for inhibition differ significantly. In spite of these differences, dibucaine numbers determined by the two methods were nearly identical. This was expected since manual assays similar to the ACA method will distinguish the normal from the dibucaine-resistant variant. Caution should be used in identifying the heterozygous genotype with the au-
Automated method, however. Under assay conditions similar to those of the ACA (but with Tris buffer at pH 7.2) the heterozygous variant is inhibited only about 10 per cent less than the normal form in contrast to the original method, which is better able to distinguish the normal from the heterozygous atypical form. Similar results have been reported using propionylthiocholine with inhibition of the heterozygous form again being only about 10 per cent less than the normal form. Our results with the automated assay suggested a similar pattern of differentiation of the heterozygous form (fig. 1), although examination of a larger population is needed to define the magnitude of these differences with the ACA.

Differential inhibition of serum cholinesterase by fluoride permits identification of a fluoride-resistant variant of the enzyme. The temperature and substrate used by the ACA are not optimal for this assay, however, and we were unable to adapt it to provide meaningful fluoride numbers.

If one accepts the limitations of the automated method for determination of dibucaine numbers, namely the relatively poor differentiation between normal and heterozygous enzyme, then the method provides a reasonable alternative to the original method with the advantages of being rapid and easily performed by any clinical laboratory that has an ACA.

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References