Biochemical Method for Chlorine Dioxide Determination

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Chlorine dioxide is a disinfectant used worldwide. In this article, a new enzymatic method for the determination of chlorine dioxide has been developed. This rapid spectrophotometric assay is able to detect from 0.2 to 4 mg/liter of chlorine dioxide. The method is based on the capacity of horseradish peroxidase to decolorize reactive yellow 17 in the presence of chlorine dioxide. The effects of several compounds on the assay have been determined. Except sodium hypochlorite, no interference was detected with 18 compounds including chlorides, sulfates, carbohydrates, amino acids, proteins, and organics. The biochemical method is faster and easier than the previous volumetric, amperometric, and colorimetric methods which are laborious and time-consuming.

MATERIALS AND METHODS

Chemicals. Horseradish peroxidase (EC 1.11.1.7, donor: H2O2 oxidoreductase) type X, bovine serum albumin, glucose, glycerol, sodium dodecyl sulfate, Tween, amino acids, N,N-diethyl-p-phenylenediamine sulfate salt (DPD), and phenol were obtained from Sigma Chemicals Inc. (St. Louis, MO). Sodium chlorite, methanol, and ethanol were purchased from Merck (Darmstadt, Germany). Mineral salts were obtained from J. T. Baker (Phillipsburg, NJ). Reactive yellow FGRL (reactive yellow 17, C.I. 18852) and other dyes were obtained from BASF (Germany).

Reagents. The reagents used were reactive yellow solution, 1 mg/ml in water and horseradish peroxidase, 40 μM. The concentration of peroxidase solution was determined by using an extinction coefficient of 91,000 M-1 cm-1 at 403 nm (14). The standard solution of chlorine dioxide was prepared according to Clesceri et al. (4) by using a gas-generating and -absorbing system. Chlorine dioxide gas is produced by the reaction of sulfuric acid and sodium chlorite. Chlorine dioxide concentration of the final solution was determined by three methods: (i) Spectrophotometrically by using an extinction coefficient of 1450 M-1 cm-1 at 360 nm (15), (ii) by the titrimetric method with ferrous ammonium sulfate and DPD as indicator (10, 12), and (iii) by the iodometric method, in which ClO2 releases free iodine from a KI solution acidified with H2SO4. The liberated iodine is titrated with a standard solution of sodium thiosul-
fate, with starch as indicator. The stock solution of ClO₂ was kept in the dark.

Procedure. Fifty microliters of reactive yellow solution (1 mg/ml) is added to a spectrophotometer cell containing 1 ml of water sample. The sample is dissolved in 60 mM phosphate buffer, pH 6.0, before performing the assay, and protected from light. The reaction is started by adding 20 µl of 40 µM horseradish peroxidase. The mixture is allowed to react for 15 min in the dark and then the mixture absorbance at 410 nm (Aᵢ) is recorded. A control mixture containing peroxidase and reactive yellow in buffer is processed in the same way as the sample and its absorbance is determined (A₀). Then, the decrease of absorbance (ΔA₄₁₀ = A₀ − Aᵢ) is calculated.

The standard curve is obtained by using dilutions of a chlorine dioxide solution, freshly prepared, in a range from 0 to 4 mg/liter in 60 mM phosphate buffer, pH 6.0. The chlorine dioxide concentration of stock solution is determined by both spectrometric and titrimetric methods.

Analytical methods. The DPD method according to Palin (10) was performed by titrating with a ferrous ammonium sulfate solution. Iodometric determination of chlorine dioxide was carried out by adding 0.5 g of KI in 100 ml of acidified sample. After 5 min, the liberated iodine was titrated with 0.001 N Na₂S₂O₃ solution with starch as the indicator. Activity of horseradish peroxidase was determined by guaiacol polymerization and monitored as the increase of absorbance at 470 nm (17). The reaction mixture contained 16 mM guaiacol and 1 mM hydrogen peroxide. Interferences to enzymatic method were estimated by comparing the assay results with and without, different amounts of several compounds in phosphate buffer. Spectrophotometric data were obtained in a Beckman DU-650 spectrophotometer.

RESULTS

Horseradish peroxidase is able to modify the reactive yellow dye in the presence of chlorine dioxide. We have assayed 26 other dyes, and reactive yellow 17 was selected because it produces decolorization in a visible-light band and no reaction was detected when peroxidase or chlorine dioxide was added alone.

Reaction rate is dependent on the amount of added peroxidase (Fig. 1) and, under our conditions (0.8 nmol/ml), after 15 min the reaction is completed. The extent of the reaction is independent of the peroxidase concentration and it is only dependent on the chlorine dioxide content (Fig. 2).

Figure 3 shows the pH effect on the assay. A complete reaction (100% response) is obtained from pH 4.5 to 6.5 after a 15-min reaction (Fig. 3a). This response is dependent on the enzyme activity. The pH affects both decolorization rate of reactive yellow with ClO₂ and peroxidase activity measured as guaiacol polymerization with hydrogen peroxide (Figs. 3b and 3c). The different activities showed different optimum pH. Peroxidase activity showed the highest rate at pH 6.0, while the highest decolorization rate was obtained at pH 5.0. However, it is possible to perform the assay at higher or lower pH, by increasing the reaction time, in order to allow for complete oxidation at lower enzyme activity.

Chloride dioxide, which is a relatively stable radical in solution, is light sensitive. It reacts with light, at a quantum yield of 2 mol/Einstein, to generate ClO· and O₂ free radicals. These radicals react via a variety of well-defined pathways to produce Cl₂, ClO₂, ClO₃, and Cl⁻ (15, 16). Thus, it is important to maintain the ClO₂...
FIG. 4. Calibration curve for chlorine dioxide determination by the biochemical method. The reaction mixture contained 50 μg/ml of reactive yellow 17 and 0.8 nmol/ml of horseradish peroxidase. ΔA_{410} was obtained after a 15-min reaction.

FIG. 3. The effect of pH on (a) chlorine dioxide determination by the biochemical method, (b) decolorization rate of reactive yellow in the presence of horseradish peroxidase and ClO₂, and (c) peroxidase activity as guaiacol oxidation in the presence of hydrogen peroxide. The buffer solutions used were 60 mM sodium acetate for pH 2–5, 60 mM sodium phosphate for pH 5.5–7.5, and 60 mM sodium bicarbonate for pH 8–12.

TABLE 1
Interferences on the Chlorine Dioxide Determination by the Enzymatic Method

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (m)</th>
<th>Method response (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>100 (±6)</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.0</td>
<td>99 (±7)</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.0</td>
<td>132 (±8)</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.0</td>
<td>81 (±1)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>0.1</td>
<td>90 (±3)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.0</td>
<td>112 (±5)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.0</td>
<td>121 (±5)</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.0</td>
<td>132 (±4)</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.1</td>
<td>102 (±3)</td>
</tr>
<tr>
<td>KCl</td>
<td>1.0</td>
<td>71 (±2)</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0</td>
<td>89 (±9)</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.0</td>
<td>121 (±3)</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>0.1</td>
<td>85 (±1)</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.0</td>
<td>81 (±3)</td>
</tr>
<tr>
<td>NaClO</td>
<td>0.002</td>
<td>D⁺</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>1.0</td>
<td>91 (±3)</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>1.0</td>
<td>97 (±1)</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>0.1</td>
<td>114 (±4)</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.1</td>
<td>97 (±4)</td>
</tr>
</tbody>
</table>

The effect of the presence of several compounds on this enzymatic method was determined. Table 1 shows only the values of the highest concentration of each compound, in which small interferences were detected.

The correlation coefficient was 0.987 ± 0.008 for four replicate curves. ClO₂ concentrations as low as 0.2 mg/liter can be determined. Thus, the assay equation is:

\[
\Delta A_{410} = (0.236 \pm 0.012)[\text{ClO}_2].
\]

The effect of the presence of several compounds on solutions in complete darkness. We found a constant ClO₂ loss of 3.06 ± 0.29% per minute, from pH 4 to 8 under light at room temperature. Nevertheless, under darkness at 4°C there was no significant ClO₂ loss (less than 1% per day).

A standard curve was obtained by using solutions containing from 0.5 to 4 mg/liter chlorine dioxide, freshly prepared. A perfect linear correlation was found (Fig. 4) by fitting the data on a linear regression with zero intercept:

\[
\Delta A_{410} = (4.23).\]

The correlation coefficient was 0.987 ± 0.008 for four replicate curves. ClO₂ concentrations as low as 0.2 mg/liter can be determined. Thus, the assay equation is:

\[
\text{Chlorine dioxide (mg/liter)} = (\Delta A_{410})(4.23).
\]

a D, dye decolorization.
TABLE 2
Comparison of Different Methods for the Determination of Chlorine Dioxide in Commercial Disinfectants

<table>
<thead>
<tr>
<th>Product</th>
<th>Chlorine dioxide found (g/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A360</td>
</tr>
<tr>
<td>ClO₂ solution</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Dichlor</td>
<td>ND</td>
</tr>
<tr>
<td>Halox</td>
<td>ND</td>
</tr>
</tbody>
</table>

* The standard deviation was obtained from four independent replications.

At lower concentrations no interference could be found. Significantly high concentrations of these compounds, up to 1 M, were not able to affect the chlorine dioxide determination by the biochemical method. From 18 tested compounds, only sodium hypochlorite was able to affect the determination of chlorine dioxide. This may be due to the presence of free and active chlorine. Surprisingly, 0.1 M hydrogen peroxide has no effect on the chlorine dioxide determination, even if it is a substrate for peroxidase.

Determination of chlorine dioxide content in a stock solution was performed. The obtained data were compared with those obtained with the absorbance, DPD, and iodometric methods (Table 2). The values obtained with our new biochemical method are consistent with those obtained with the titrimetric methods. On the other hand, two commercial preparations were tested. Dichlor, a commercial product, labeled as containing 10% (minimum) of active chlorine (equivalent to 190 g/liter of ClO₂), shows 104% of the chlorine dioxide, while Halox E-100 (Halox American, Burlingame, CA), claiming 6% of active chlorine, shows values of 129 g/liter of chlorine dioxide or 6.78% active chlorine.

**DISCUSSION**

We have developed a new spectrophotometric method for chlorine dioxide determination. This method is much easier and faster than volumetric, amperometric, and colorimetric methods, which are laborious and lack speed, sensitivity, or specificity (10-12).

This method is based on the catalytic activity of horseradish peroxidase. Monochlorodimedone has been transformed to dichlorodimedone by the action of horseradish peroxidase and sodium chlorite (18). Chlorite is disproportionated catalytically by horseradish peroxidase to form chlorine dioxide and chlorine ion (19), and the product chlorine dioxide is responsible for the chlorination of monochlorodimedone. The enzyme species formed seems to be compound II as shown by transient intermediate spectra (19). This is a direct oxidation of native horseradish peroxidase to compound II. Oxidation of compound II to compound I is performed by chlorine dioxide (20). Reaction of horseradish peroxidase at neutral pH produces mainly compound I and small amounts of compound II (20). Thus, in our case the compounds I and II may be involved in the reactive yellow decolorization. Other hemoproteins, such as hemoglobin, are able to modify catalytically phenols in the presence of sodium chlorate (21).

This method has almost no interference with high concentrations (up to 1 M) of several compounds (Table 1). At compound concentrations lower than those shown in Table 1 no significant interference could be detected. The compounds tested included chlorides, sulfates, phosphates, organic compounds, detergents, organic solvents, and ions, such as potassium, sodium, and magnesium. Ethanol, MgCl₂, SDS, and Tween at 1.0 M produced a precipitate, making the determination assay impossible. Hypochlorite ion affects seriously the ClO₂ determination. Very low hypochlorite concentrations (<1.7 mm) are able to decolorize the yellow dye without peroxidase. This nonenzymatic decolorization could be used as an indicator for the presence of ClO⁻ ion. Nevertheless, the analysis of a sample containing ClO₂ and hypochlorite can be carried out by a conventional method for chlorine determination and using the pH-dependent reduction of chlorine species. The total available chlorine (free plus combined) is determined at pH 12 in which ClO₂ is converted to chlorite and chlorate. In a second titration, ClO₂ is estimated at pH 2 in the presence of KI (12).

In conclusion, the new biochemical method for determination of chlorine dioxide constitutes a valuable tool for routine determination in water treatment plants and other industrial processes. This method is much more rapid, less laborious, and less time-consuming than the titrimetric or amperometric methods. With this accurate method it is possible to detect from 0.2 to 4.5 mg/liter of ClO₂ and it is not affected by normal substances present in water. The biochemical determination of chlorine dioxide in commercial disinfectants and freshly prepared solutions showed consistent results when compared with other two titrimetric methods.

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**REFERENCES**


