Effects of Temperature and Relative Humidity on Biological Indicators Used for Ethylene Oxide Sterilization

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A study was made to determine the effects of temperature and moisture on the D-value of a common biological indicator. Relative humidity (RH) was varied between 10 and 70% in increments of 10%, and temperature was varied between 30 and 70°C in increments of 10°C. Temperature was found to have a pronounced effect on the D-value. At 60% RH, the D-value varied from 15.0 min at 30°C to 1.1 min at 70°C. When RH was plotted against the average D-value at the various temperatures, the temperature curves at or above 50°C were more erratic and the RH had a significant effect. The study showed that temperature and RH must be controlled if biological indicators are to be properly calibrated for use in ethylene oxide sterilization.

Biological indicators (BIs) have been used for many years to indicate adequate sterilization by ethylene oxide (EO). Many BIs are considered to be more inconsistent and unreliable than chemical indicators, even though most chemical indicators show only exposure effects and do not indicate time of exposure or concentration of the sterilant. When properly calibrated, BIs are integrators of the many physical and chemical parameters of a sterilization process. BIs that have been tested against known conditions of relative humidity (RH), temperature, and gas concentrations are said to be "calibrated" for those conditions.

BI performance characteristics are becoming more important to the Food and Drug Administration because of the trend toward parametric (process) release of sterilized products and the use of BIs to verify adequate sterilization. A biological indicator evaluation resistometer (BIER) was developed by the Food and Drug Administration to test BIs used for EO sterilization, and a survey of the performance characteristics of the BIs from users and manufacturers was completed (5). Approximately 30% of the BIs surveyed did not pass the United States Pharmacopeia, 19th revision (USP XIX; 11), criteria as tested, probably because the test conditions used by some manufacturers differed from those of the Food and Drug Administration; i.e., different temperature and RH, different gas concentrations, or both were used. Both the Food and Drug Administration and the manufacturers recognized that standard test conditions must be established.

Kereluk et al. (1-4) described the effects of EO concentration and humidity or water activity on the bacterial spores of Bacillus subtilis subsp. niger (Ft. Detrick strain). Moisture was less critical than the EO concentration. Tests by Reich et al. (9) in a steam BIER showed that storage conditions significantly changed the decimal reduction time (D-value) and viability of Bacillus steaerothermophilus and that the variability was due in part to the water activity or moisture level during storage.

The effects of temperature and moisture levels on the D-value of microbial spores used for EO monitoring should be clearly defined to the BI user or manufacturer. With recently developed equipment, precise moisture and temperature studies can provide the required data. This study determined the effects of temperature and moisture on the D-value of a commonly used BI.

MATERIALS AND METHODS

Spordex BIs (American Sterilizer Co., Erie, Pa.) were used to evaluate the effect of various temperatures and moisture levels. The BIs were paper strips inoculated with approximately 6.0 × 10⁶ colony-forming units of B. subtilis subsp. niger (globigii). Resistance data provided with the BIs indicated a D-value of 3.0 min at 600 mg of EO per liter at 54°C and 60% RH. The prescribed D-value was confirmed in the test system used for this study. The sterilant gas used was 88% dichlorodifluoromethane (Freon) and 12% EO (Pengas) (Pennsylvania Engineering Co., Philadelphia, Pa.).

The BIER used for this study consisted of a chamber 36 in. (ca. 89 cm) long and 10 in. (ca. 25 cm) in diameter (American Sterilizer Co.), modified to maintain temperatures to ±1°C. The temperature was controlled with an RFL laboratory temperature controller (RFL Industries Inc., Boonton, N.J.) and was monitored with an L & N Speedomax recorder (Leeds and Northrup, North Wales, Pa.). The chamber was con-
nected to two Miran II infrared analyzers (Wilks Scientific, South Norwalk, Conn.) with 0.25-in. (ca. 0.6-cm) stainless-steel tubing. A pump circulated the gases in the chamber and through the analyzers in a closed loop. One infrared analyzer monitored EO; the other monitored RH. BIs were exposed to the RH level being tested for 30 min before exposure to EO.

Figure 1 shows a typical profile for a BIER run. The BIs were placed in the chamber, a vacuum of 26 in. of Hg was drawn, and steam was introduced to the desired moisture level. Dry air was allowed into the chamber to disperse the moisture evenly and to help drive the moisture into the BI containers. Humidification proceeded for 30 min before evacuation to 26 in. of Hg. The chamber was rehumidified to the desired moisture level, and 600 mg of EO per liter was immediately introduced. A heat exchanger heated the EO gas mixture to reduce the temperature drop caused when the gas was introduced into the chamber. Vacuum was again drawn to 26 in. of Hg at the end of the exposure time, and the chamber was flushed with ambient air before BI removal.

Exposure levels of EO were 600 mg/liter for all analyses. RH was varied between 10 and 70% in 10% increments, except when the temperature was increased to 70°C. At 70°C, humidities above 60% were not reliable because of the formation of condensate in the gas transport lines; analysis was therefore conducted at RH levels of 10 to 40 and 60%.

Temperature was varied between 30 and 70°C in 10°C increments. Although temperature was stabilized at the desired temperature, it dropped approximately 6°C when the vacuum was drawn. However, it was stabilized again within 5 min after the readmission of air or EO gas mixtures.

RESULTS

Figure 2 shows the effects of RH on the survival curves at various temperatures. The data indicate that, as the temperature was reduced from 70 to 30°C, the effect of RH became more pronounced. Figure 3 compares the D-value (determined as an average of slopes between individual data points) (6–8, 10) for 30 and 60% RH. The temperature had a pronounced effect on the average D-value at 60% RH, varying from 15.0 min at 30°C to 1.1 min at 70°C. A D-value of 3 min, determined at USP XX (12) conditions, fell on the curve at 54°C and 60% RH. Temperature had less effect on the average D-value at 30% RH and ranged from 8.1 min at 30°C to 2.1 min at 70°C.
Figure 4 shows the effect of RH on the average D-value at the temperatures tested. Curves for temperatures at or above 50°C became nearly straight and vertical above 30% RH, indicating that RH had little or no effect on the D-value. Curves for temperatures below 50°C were more erratic and RH had a significant effect, with D-values generally increasing as RH increased. The D-value of 3 min for the spore strips, determined at USP XX (12) test conditions, fell between the 50 and 60°C lines, indicating that the test conditions proposed in USP XX are adequate to provide reliable and predictable D-values.

**DISCUSSION**

Data in this study indicate that manufacturers using test temperatures below 50°C must maintain greater control of RH to provide consistent test parameters and determine D-values accurately. At test temperatures above 50°C, RH conditions between 30 and 70% have little effect on the D-value.

Each test parameter has significant and varying effects on the D-value. If resistance data collected under other than USP XX standard test conditions are to be compared with USP XX specifications for BIs, the test conditions must be validated by comparing both methods simultaneously. Testing BIs without validating the test method has resulted in claims by some manufacturers that the resistance of their BIs is consistent with USP XX specifications when, in fact, it may not be. Tests conducted with low RH (10 to 30%) show a higher D-value; at temperatures below 50°C, D-values increase rapidly as RH increases. The data show that the USP XX test conditions provide a sufficient challenge to ensure that BIs are meaningful indicators of sterilization conditions. The determined D-value of 3 min (Fig. 3 and 4) was within the most stable temperature and RH ranges.

Because RH is difficult to control in a test cycle, D-value determinations should be conducted at or above 50°C and near the midrange of RH stability (50%) to minimize the effect of RH and provide stable test conditions. With properly calibrated BIs, pharmaceutical and medical device manufacturers can accurately evaluate sterilization processes and meet good manufacturing requirements.

**LITERATURE CITED**


