A fluorescence bioassay to detect residual formaldehyde from clinical materials sterilized with low-temperature steam and formaldehyde

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Abstract

A microtiter plate toxicity test based on fluorescence was developed to determine the residual concentration of formaldehyde on medical items after LTSF sterilization. The residual formaldehyde on eight common materials, some of which are used in different clinical instruments and devices were analysed after sterilization with LTSF. Formaldehyde residues were detected on cotton, filter paper, natural rubber, PVC, and silicone-coated latex, but not on polyurethane, silicone or glass. Formaldehyde never exceeded the recommended maximum concentration on clinical devices of about 5 μg/cm². The results were compared with those obtained by means of a chemical method, the correlation being good (R² = 0.9396).

The biological method proposed here is fast and can be automated, which means that it could be used as a screening method when there are doubts as to the accumulation of residues on clinical materials or instruments that are going to be sterilized with LTSF.

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1. Introduction

Many clinical instruments and devices contain, or are made of, heat-labile materials that cannot be sterilized by autoclaving. Low-temperature steam and formaldehyde (LTSF) sterilization is one of the available alternatives most used in many European countries, although, due to the danger posed by formaldehyde, there were some doubts at first as to the advisability of using this system.

In 2004 the USEPA and the International Agency for Research on Cancer (IARC) have declared formaldehyde as a carcinogen. In addition, it is known to have some detrimental side effects, including respiratory dysfunction, contact dermatitis and possible allergic reactions [1]. Nonetheless, the most modern employed LTSF sterilization systems use solutions containing formaldehyde at low concentrations (2%), which are supplied in disposable bags that do not require manipulation by the personnel in charge of sterilizing materials as they are automatically dosed. Moreover, the requirements and testing of these sterilizers for medical purposes were published in 2003 by the European Committee for Standardization on the European Standard EN 14180.
but their usefulness and safety have been well documented for some time now [3–6].

Once the sterilization process has been completed, these new systems do not require additional aeration, nor do they need an isolated installation or special precautions during operation. Furthermore, they can be monitored by commercially available chemical and biological controls. As required by the European standard EN 14180, with the newest LTSF sterilizers on the market it is possible to validate the method and quantify residues that might have been left on sterilized materials.

On the other hand, the medical industry is continuously developing new materials, some of which are incorporated into clinical instruments and devices that have become increasingly more varied over the years. In the majority of cases, the capacity of these materials to absorb formaldehyde residues and, therefore, the effect these residues may have on patients are still unknown. So the objective of this study was to adapt a bacterial toxicity test, initially developed for detecting disinfectant residues released by disinfected materials, as methods of screening that could be used to determine the formaldehyde residues absorbed by different materials after LTSF sterilization. The developed assay, based on the bacterial growth-inhibition, can be fully automated and requires a small sample size for testing formaldehyde concentrations in the μg/cm² range as those accumulated on some materials sterilized with LTSF. Bacterial growth-inhibition was determined by a fluorometric method in microtiter plate, adapted from a previously described bacterial toxicity assay performed in a test tube [7]. The results of this microtiter-based fluorescence bioassay were then compared with those of a chemical desorption test.

2. Materials and methods

2.1. Carriers

The materials used as carriers were cut into fragments of the same size with a scalpel in sterile conditions. The eight items were as follows: polyvinyl chloride (PVC) and silicone (SI) tubes, both with an internal diameter (ID) of 2 mm, an outer diameter (OD) of 4 mm, and a length of 20 mm, from Brand (Germany); polyurethane (PUR) tubes (2.4 mm ID, 3.8 mm OD, and 20 mm length) from Braun Melsungen (Germany); glass tubes (7 mm ID, 9 mm OD, and 10 mm length) from Albus (Spain); silicone-coated latex tubes (2.5 mm ID, 7 mm OD, and 20 mm length) from Kendall Company (USA); natural rubber (30 × 30 × 2 mm); and 100% cotton material from hospital cloths and standard filter paper (90 g/cm² and 0.2 mm of thickness), both of 30 × 30 mm. The measurements for the calculation of the surface of the carriers were taken with an electronic digital calliper (Comecta, Spain).

2.2. LTSF sterilization

A #130F sterilizer (Matachana, Spain), installed at the Central Sterilisation Service of University Hospital of Malaga (Spain), was used. The sterilization cycle consisted of six phases: pre-vacuum; pre-pulses of steam; formaldehyde feed; sterilization; washing pulses; and air pulses. A detailed description of each one of the LTSF sterilization phases was published recently [8]. The sterilization cycles employed in this study were performed with a programme using steam at 60 °C, and a 2% concentration of formaldehyde, the sterilization processes being carried out at 123 mbar for 120 min.

Sterilization monitoring was performed according to international and European Standards EN 14180. The monitoring system Attest™ (3M, Spain), containing spores of Geobacillus stearothermophilus (reference 1262), was used as the biological indicator [9,10]. Following the recommendations of the manufacturer of the LTSF sterilizer, three types of indicator were used as chemical controls: lot control, indicator inside and detector spots, supplied by Albert Browne Ltd. (Leicester, UK).

As is normal in routine hospital practice, the items used as carriers in the experiments were introduced into Esteripapel™ bags (Soplaril Hispania, Spain) for LTSF sterilization. The presence of formaldehyde residues on samples submitted to five sterilization cycles was analysed, as were 10 replicates of each one of the tested materials in each cycle. Likewise, the corresponding controls were used to check in each cycle whether the sterilization process had been carried out properly.

2.3. Chemical desorption test

Once sterilized with LTSF, the carriers were immediately taken out of their sterilization bags, and five samples of each type of test material introduced separately into test tubes containing 5 ml of 0.2 N NaOH. After 30 s of shaking with a vortex shaker, the tubes were left to settle for 8 h. The same procedure was then followed with the items used as controls, which had not been previously sterilized with LTSF. Next, 1 ml of this solution was mixed with 10 ml of chromotropic solution and the concentration of residual formaldehyde was determined by regression using an absorbance calibration curve obtained from known concentrations of formaldehyde as is described in EN 14180.

2.4. Fluorescence bioassay

2.4.1. Standard bacterial suspension preparation

An overnight culture of Escherichia coli W3110 Thy⁻ (obtained from the Spanish Collection of Culture Types, Burjasot, Spain) was grown in 10 ml of Vogel–Bonner minimal medium (VB), with thymine (50 mg/l) and
glucose (20 g/l), at 37 °C for 18 h, and then centrifuged, the harvested cells being washed and suspended in 10 ml of VB medium (without thymine or glucose). The optical density of this suspension at 600 nm (OD₆₀₀) was determined in 1 cm pathlength cuvettes and adjusted to 0.5 ± 0.01. A 10% v/v dilution in VB medium of this suspension (which corresponds approximately to 10⁸ cells in ml), prepared immediately before use, was used as the standard bacterial suspension in all experiments.

To select this standard suspension, the intensity of the fluorescence emitted by different sizes of bacterial inoculum due to the glucuronidase activity of *E. coli* was previously measured. In each one of the individual wells of a polystyrene 96-well microtiter plate with a flat bottom and lid (#P612F96, Bibby Sterilin Ltd, UK), 150 µl of VBTM medium (VB medium containing thymine, 50 mg/l, and 4-methylumbelliferyl-β-D-glucuronide, 100 mg/l) was added, together with 50 µl of one of the five different sizes of inoculum (between 1 and 50% v/v) obtained from a bacterial suspension OD₆₀₀ (0.5 ± 0.01) in VB medium. Subsequently, the plate was incubated at 37 °C and the fluorescence measured at 1 h intervals as described below in the following section.

### 2.4.2. Toxicity testing

A previously described fluorescence bioassay [7] was adapted as a microplate technique to facilitate its use as a routine, automatable screening method in clinical work. For the desorption of formaldehyde residues, five carriers of each one of the materials sterilized with LTSF were introduced separately into sterile test tubes containing 5 ml of VB medium and then shaken for 30 s and allowed to settle at room temperature for 8 h. This VB solution and ½ and ¼ dilutions in VB medium were used directly as samples in toxicity testing, the same procedure being followed with the unsterilized items used as controls.

For toxicity testing, polystyrene 96-well microtiter plates, such as the one previously indicated, were used. One hundred microlitres of each sample, or of its dilutions (½ and ¼), was placed separately in the wells of the microtiter plates. In each assay, three replicates of each sample, or their dilutions, were analysed along with their respective controls (samples that had not been sterilized in an autoclave with LTSF), three wells being used as blanks. Simultaneously, a calibration curve was prepared in other wells of each microtiter plate assay containing 100 µl of double dilutions of formaldehyde (Sigma—Aldrich Chemie, Germany) in VB medium. Subsequently, 50 µl of VBTM was added to each well (samples and calibration curve) using a multichannel pipette, and the 96-well plate was then closed and placed on a microplate shaker with a throw of 1.5 mm, for 2 min. Finally, all the wells, except for the blanks, were inoculated with 50 µl of standard bacterial suspension, covered and incubated with shaking at 37 °C for 3 h. Sterile VB medium (50 µl) was added to the wells used as blanks. Fluorescence was then measured using a Kodak EDAS 290 image capture system with a 590 nm filter, and Kodak 1D Image Analysis Software. The average intensity of the pixels (AIP) for each well measured with the help of the software’s ROI (Regions of Interest Measurements) application was used as hard data for analysis.

Formaldehyde concentration in samples was determined from the calibration curve in function of the percentage of inhibition versus control (% Inhibition) obtained from values AIP. This % Inhibition was calculated from the difference between values AIP in samples and controls after 3 h incubation in the fluorescence bioassay:

\[
\% \text{Inhibition} = \frac{(AIP_c - AIP_b) - (AIP_s - AIP_b)}{(AIP_c - AIP_b)} \times 100
\]

where c, s and b are the average AIP values in controls (unsterilized materials), samples (materials sterilized in formaldehyde), and blanks, respectively. The inhibition values of the samples were calculated from the sigmoidal concentration— inhibition curves fitted by probit analysis using the maximum-likelihood solution [11].

### 2.4.3. Statistics

A comparison of means test based on the Student’s *t* test was conducted to calculate the differences between the residual concentrations of formaldehyde as estimated in the chemical and fluorescence tests. Differences were considered statistically significant at *p* ≤ 0.05 level of significance.

### 3. Results

To standardize test operating conditions, *E. coli* glucuronidase activity for different inoculum sizes was previously determined. Fig. 1 shows glucuronidase activity trends of the inocula tested. As can be seen, the inocula reached a maximum value after 3–5 h of incubation, those with the greatest amount of microorganisms reaching maximum fluorescence in the shortest time. Bearing in mind these results, 3 h of incubation and the inoculum 10% v/v as the standard inoculum, were chosen as the best operating conditions for the fluorescence bioassay. The criteria used in the establishment of these operating conditions included the choice of the inoculum with the smallest number of microorganisms and capable of emitting fluorescence both perfectly visible to the naked eye under UV illumination and statistically quantifiable in the described test conditions in the shortest time possible.
Fig. 2 shows the effect of formaldehyde on the glucuronidase activity of *E. coli* after 3 h of incubation, using the standard bacterial suspension (inoculum 10% v/v). The percentage of inhibition showed a linear trend in the 0.6–10 µg/ml range of formaldehyde. This range was chosen as a calibration curve to be employed in all the microtiter plates in the fluorescence.

The results of the chemical test and the fluorescence bioassay for determining residual formaldehyde from materials sterilized with LTSF are shown in Table 1. As can be seen from these results, formaldehyde concentrations greater than 1 µg/cm² were only detected on two of the test materials (cotton and paper). As regards the rest of the materials, concentrations were much smaller and, in the case of three (silicone, PUR and glass), both of the tests failed to detect any formaldehyde residues at all. Furthermore, regarding the methods used to detect residues, formaldehyde concentrations detected by both methods on the different test materials were very similar. Although average formaldehyde concentrations were always higher when using the chemical method than the fluorescence bioassay, with the exception of the samples of PVC, the differences were in no case statistically significant (p ≤ 0.05).

Measurements of formaldehyde residues showed a good correlation between fluorescence and chemical test results, with a linear fitting curve and an $R^2 = 0.9396$ (Fig. 3).

4. Discussion

Our method enables to estimate the presence of formaldehyde residues on different types of materials sterilized with LTSF, based on the toxicity of these residues on the viability and killing of an indicator microorganism. As described previously, it is based on the amount of fluorescence emitted, resulting from the

<table>
<thead>
<tr>
<th>Item</th>
<th>Chemical test</th>
<th>Ratio</th>
<th>Fluorescence test</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotton</td>
<td>1.51 ± 0.18</td>
<td>0.85</td>
<td>1.27 ± 0.20</td>
<td>0.76</td>
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<tr>
<td>Filter paper</td>
<td>1.78 ± 0.16</td>
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<td>1.67 ± 0.290</td>
<td>1</td>
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<tr>
<td>Glass</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Natural rubber</td>
<td>0.09 ± 0.03</td>
<td>0.05</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polyurethane</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polyvinyl chloride</td>
<td>0.28 ± 0.10</td>
<td>0.16</td>
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<td>0.18</td>
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<tr>
<td>Silicone</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Silicone-coated latex</td>
<td>0.53 ± 0.13</td>
<td>0.30</td>
<td>0.49 ± 0.14</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Five replicates were tested and compared with controls in both assay.

a Results in chemical test and fluorescence test in µg/cm² ± standard deviation.

b Concentration of formaldehyde in the sample relative to that filter paper.

![Fig. 1. *E. coli* glucuronidase activity estimation for different inoculum sizes on microtiter plates. Comparison between fluorescence emission measure curves for 50% v/v, 25% v/v, 10% v/v, 5% v/v, and 1% v/v inoculum. Dots represent the mean of five fluorescence readings.](image1)

![Fig. 2. Formaldehyde calibration curve obtained from the fluorescence bioassay. % Inhibition was plotted versus log formaldehyde concentrations. The dots represent the average percentage of inhibition of the glucuronidase activity of *E. coli* in relation to the controls, and the continuous line, the data fit. Results are shown as means of five separate experiments.](image2)
glucuronidase activity of E. coli, proportional to the increase in the number of viable microorganisms [12]. The fluorescence method described in this paper offers important advantages over the original method initially developed by the authors to detect residual bacterial activity from the use of disinfectants on hard surfaces by means of a manual procedure [7]. More recently, this assay was used to detect the same effect on clinical materials, such as probes or catheters [13].

As can be seen in Table 1, the average residual concentrations measured by the fluorescence bioassay in the five processes of LTSF sterilization showed a good reproducibility, similar to that of the chemical test, as was confirmed by the variation coefficient (between 15 and 39% in the fluorescence bioassay and 9–37% in the chemical test).

Residual formaldehyde detected on materials that come into close contact with patients’ mucous membranes may pose a health risk. Although maximum limits for the presence of formaldehyde residues on clinical materials have yet to be established, in EN 14180 the mean safety limit value is set at 200 µg, and it is generally accepted that its residual presence should not exceed 5 µg/cm² [14]. Although some of the materials included in this study, such as PVC or natural rubber, are not regarded as compatible with formaldehyde gas sterilization, both in this study and in other previous ones, none of them exceeded this level [7,14,15]. The other materials on which residues were detected at a concentration higher than 1 µg/cm² were included in the study as reference materials with high porosity, although in routine practice none of them need sterilization and, should they require it, other methods are used. On the other hand, there is little or no information about formaldehyde residues on various materials following LTSF sterilization [16]. In addition, differences in the processes of sterilization — temperature, total of vacuum and steam pulses or air pulses — [5], the concentration of formaldehyde used in the process or various aspects as form, thickness and area of the studied surfaces, makes it difficult to compare the results. In a recent study [16], residual formaldehyde on several materials and medical devices was measured following LTSF sterilization. Except the filter paper used as reference, the other materials were not similar in size, forms or composition that included in our study. The process of sterilization followed also was different. The amount of formaldehyde residue varies according to the surface area and other factors, and should be verified when material LTSF sterilization is performed. However, further evaluation using standard European EN1418 and identical materials is required to confirm the results.

The advantages micro methods have over other methods based on cellular growth, such as OD increase or microorganism plate count, as regards the speed with which they estimate the effect of toxic substances on microorganisms and provide reliable results, have already been widely studied [17,18]. The microtiter fluorescence bioassay described here, with its good correlation with the chemical test (Fig. 3), can be used to analyse simultaneously a large number of samples with a minimum of user intervention, being a method easy to automate and one that allows results to be obtained in real time. In addition, although an image capture and digitalization system and scientific image analysis software have been used in the experiments described in this paper, the concentration of residues with toxic activity can also be measured semi-quantitatively, comparing with the naked eye and with the help of a UV lamp the fluorescence emitted by the wells containing the samples with that emitted by the wells with known concentrations of formaldehyde, corresponding to the calibration curve. This semi-quantitative reading only provides a rough estimate of the concentration of the residues on sterilized materials, but it could be used as a rapid control for estimating these residues before being calculated accurately using image digitalization. Evidently, this semi-quantitative determination should be carried out with adequate safety measures to avoid the harmful effects of exposure to UV radiation, but at any rate it provides a quick, first estimate of the concentration of formaldehyde residues on samples.

In this new microtiter-based fluorescence bioassay, after adding the samples and the known concentrations of formaldehyde for the calibration curve, the reaction mixture and the bacterial inoculum can be added simultaneously to all the wells using a multichannel pipette. This also allows a greater control over the reaction time and simultaneous reading of the fluorescence in all the samples and their respective controls, as well as those corresponding the calibration curve. On having miniaturized the assay, in addition to these advantages, it is worth mentioning that it requires less manipulation and uses considerably fewer reagents.

![Graph: Correlation between residual formaldehyde on cotton, natural rubber, paper and PVC in the fluorescence bioassay and in the chemical test. The dots represent experimental data for five replicates.](image)

\[ y = 0.8803x + 0.032 \]

\[ R^2 = 0.9396 \]
Furthermore, results can be obtained immediately by comparing ocularly the fluorescence emitted by the samples with that emitted by the wells containing different concentrations of formaldehyde for plotting the calibration curve, as mentioned earlier.

In recent years, low-temperature steam and formaldehyde sterilization has been improved while its use had been extended to a number of European countries [19]. Modern sterilizers, such as the one used in this study, have passed all the environmental controls with flying colours [20]. In some cases, the recommendations for avoiding the sterilization of certain materials like latex or PVC with LTSF have nothing to do with retained residues but with their deterioration as a consequence of repeated sterilizations [21,22]. Owing to the continuous introduction of new materials in the manufacturing of medical instruments, and to the dearth of information about the potential toxic side effects of disinfectant residues on these materials, it seems appropriate that methods, which are both automatable and easy to use in laboratories, should be made available to detect such residues. This study shows that a microtiter-based fluorescence bioassay for determination of residual formaldehyde can be conducted quickly and reliably using conventional equipment available in the majority of laboratories. Formaldehyde residues at a low concentration of micrograms per cm$^2$ can be measured without the samples having to be pre-treated, thus constituting a quick, cheap screening method.

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