INTRODUCTION

Disinfectants are chemicals that can destroy or inhibit the growth of harmful microorganisms in non-sporulated or vegetative states [1]. They are diverse compounds with a variety of chemical structures. Glutaraldehyde and benzalkonium chloride are two examples of active substances used as disinfectants. Glutaraldehyde is used as a 2% aqueous solution, particularly in hospitals as a common means of sterilizing medical equipment [1]. Benzalkonium chloride is also widely used in disinfectant in hospitals, farms, and food & beverages at a concentration of 0.13%. It is a cationic surfactant and is widely used as a disinfectant because of its ability to denature proteins. In hospitals, a 10% solution of the benzalkonium chloride is usually diluted to 0.05–0.1% and used for various types of disinfection [2].

Glutaraldehyde and benzalkonium chloride levels are determined by titration [3] where the measurement of the endpoint can be inaccurate. Therefore, it would be desirable to develop a faster, easier, and more accurate method to determine glutaraldehyde and benzalkonium chloride concentrations using high-performance liquid chromatography (HPLC).

Several quantitative HPLC assays for benzalkonium chloride have been reported, but they are time-consuming (10–20 min) and use relatively long detection wavelengths ranging from 240 to 270 nm which reduce their sensitivity [4]. Analysis of glutaraldehyde by gas chromatography and HPLC has been compared and showed that HPLC produced good linearity, repeatability, and low detection limit but the separation was time consuming (10–15 min). Hence, it is desirable to develop an assay for benzalkonium chloride and glutaraldehyde that is faster, selective, and accurate using HPLC with ultraviolet (UV) visible detection.

MATERIALS AND METHODS

Samples

Samples used were four disinfectants commercially available: A, B, C, and D. Samples A and B contain benzalkonium chloride, while C and D contain glutaraldehyde.

Chemical material

The chemicals used were 50% benzalkonium chloride (Sigma-Aldrich), glutaraldehyde 50% (Sigma-Aldrich), acetonitrile pro HPLC (Merck), glacial acetic acid (Merck), sodium acetate (Merck), distilled water (Ikapharmindo), 85% phosphoric acid (Merck), and 2,4 dinitrophenylhydrazine (DNPH, Sigma-Aldrich).

Chromatographic conditions

An LC 20AT HPLC system (Shimadzu) equipped with pumps, Sunfire® C8 columns (250 mm column length, 4.6 mm inner diameter, and 5 μm particle size), and UV-visible detector (SPD 20 A, Shimadzu) set at 365 nm for glutaraldehyde and 210 nm for benzalkonium chloride.

Method

Preparation of DNPH solution

About 50 mg of DNPH was weighed into a 50 mL volumetric flask and 0.25 mL of 85% phosphoric acid added followed by acetonitrile q.s. 50 mL. The sample was shaken until dissolved.

Preparation of glutaraldehyde standard solution

Nearly 250 μL of 50% glutaraldehyde standard was diluted to 100 mL with acetonitrile.
Preparation of standard benzalkonium chloride solution
About 200 μL of standard 50% benzalkonium chloride solution was pipetted into a 10 mL volumetric flask and diluted to the limit mark using a mobile phase to produce a standard 1% solution of benzalkonium chloride. This was also diluted 10-fold further.

Preparation of 0.6 M acetate buffer solution pH 4
Nearly 0.6 M acetate buffer solution pH 4.6 was prepared by dissolving 5.4 g of sodium acetate in 50 mL pH adjusted to 4.6 with glacial acetic acid and then diluted with water to 100 mL.

Glutaraldehyde derivatization optimization
About 50 μL of standard glutaraldehyde solution was placed into 5 mL amber flasks, and 1, 2, 4 mL of DNP solution was added. Acetonitrile was then added q.s. 5 mL, and the samples were shaken to homogenize. Each solution (20 μL) was injected into the HPLC and analyzed.

Glutaraldehyde derivatization
For optimal derivatization, 2 mL of DNP solution was pipetted into a 5 mL amber flask and 50 μL of 50% glutaraldehyde standard solution was added. Acetonitrile was added q.s. 5 mL.

Mobile phase selection
Standard solutions of benzalkonium chloride and derivatized glutaraldehyde were each injected (20 μL) into the HPLC and analyzed isocratically at a 1.0 mL/min flow rate using the buffer conditions as shown in Tables 1 and 2.

Optimum analysis conditions were assessed based on peak retention time (tR), peak sharpness, tailing factor (Tf), resolution (R), number of theoretical plates (N), and height equivalent to a theoretical plate (HETP).

Flow rate selection
Standard solutions of benzalkonium chloride and derivatized glutaraldehyde were each injected (20 μL) onto the HPLC and run at flow rates of 0.8, 1.0, or 1.2 mL/min.

Furthermore, the most optimum analysis conditions were assessed based on peak tR, peak sharpness, Tf, R, N, and HETP.

System suitability test
Standard solutions of benzalkonium chloride and derivatized glutaraldehyde solutions were injected (20 μL) onto the HPLC and run at the optimized flow rate and in the optimized mobile phases up to 6 times. Peak areas were integrated, and a relative standard deviation (RSD) of 2% or less was considered acceptable.

Validation of analytical methods
Glutaraldehyde calibration curve
5, 40, 50, 30, 40, and 50 μL were placed into 5 mL amber flasks containing 2 mL DNP and acetonitrile q.s. 5 mL and shaken to homogenize. 20 μL of each standard solution was analyzed.

Benzalkonium chloride calibration curve
1% benzalkonium chloride solution was diluted to 20, 50, 100, 200, 300, and 400 μg/mL. 20 μL of each standard solution was analyzed.

Detection limit test (LOD) and quantitation limit (LOQ)
Detection and quantitation limits can be calculated statistically by linear regression of calibration curves. The calculation result will be equal to the value of b in the equation (y=a+bx), whereas the SD of the blank is equal to the residual SD (Sy/x)[9].

Selectivity
Selectivity was assessed using standard solutions. 20 μL of each solution was injected into HPLC under the optimized analysis conditions. The observation was conducted to find if there was a difference in tR between samples and standards and if there was other peaks appeared at the time of retention of benzalkonium chloride and glutaraldehyde in the sample solution.

Accuracy and precision
Sample was prepared using the same procedure used for standard curves so as to produce low, medium, and high samples containing 80, 100 μg/mL, and 120 μg/mL of benzalkonium chloride, respectively. Similarly, 8, 10, and 12 μg/mL samples of glutaraldehyde were also prepared. Each sample was produced in triplicate and 20 μL of each analyzed.

Sample preparation
Samples containing benzalkonium chloride (A and B)
A 1.5 mL aliquot sample solution then inserted into a 100 mL measuring flask, then dissolved and diluted with a mobile phase up to the limit mark, shake, and homogenized. 20 μL was analyzed by HPLC.

Samples containing glutaraldehyde (C and D)
1.5 mL sample was diluted into 100 mL with acetonitrile and shaken to homogenize. 50 μL of the solution was inserted into a 5 mL chrysanthemum that has been filled 2 mL DNPH and then homogenized. 20 μL was analyzed by HPLC.

Sample analysis
Each sample was treated as described above. 20 μL aliquots were analyzed by HPLC and peak areas recorded. Injections were performed in duplicate.

RESULTS AND DISCUSSION

Method optimization
Glutaraldehyde derivatization
Derivatization in HPLC methods is required to transform the analyte into a form that can be detected with the required sensitivity and selectivity [10]. Glutaraldehyde is a compound with no chromophore group so that it must be derivatized with DNP. DNP was titrated to determine optimal derivatization conditions. Thus, 1, 2, or 4 mL of DNP was added to the glutaraldehyde standard. The largest peak area was produced with 2 mL of DNP, and this also yielded the highest resolution than the other volumes.

Selection of mobile phase composition
Acetonitrile-acetate pH 4 at various ratios was used as the mobile phase for the analysis of benzalkonium chloride and to determine tR, HETP, N, and Tf (Table 3). Based on these results obtained, the 75:25 ratio...
was chosen as the mobile phase for benzalkonium chloride because it produced the best separation and largest peak area.

For glutaraldehyde analysis, various ratios of acetonitrile:water were used as the mobile phase to determine the same parameters. The 75:25 ratio was chosen because it produced a higher resolution than the 80:20 and a shorter retention than 60:40.

**Flow rate selection**

After optimizing the mobile phase, flow rate optimization is carried out with variations of 0.8, 1.0, and 1.2 mL/min for both the analytes (Tables 4 and 5). Based on the results, 1.2 mL/min was chosen for benzalkonium chloride because it produced the fastest elution while exhibiting good separation (Table 4). The same flow rate was chosen for glutaraldehyde because of the shorter retention time and the resolution did not vary substantially from the other flow rates (Table 5).

**System suitability test**

The suitability test of the system was performed 6 times by successive injection of standard benzalkonium chloride solution using an acetonitrile-acetate buffer phase (75:25) at a flow rate of 1.2 ml/min and a glutaraldehyde standard with an acetonitrile-water phase (75:25) at flow rate of 1.2 ml/min. The benzalkonium chloride system produced an RSD of 0.55%, while the glutaraldehyde method produced an RSD of 0.94%, both of which were below the maximum requirement of 2%.

**Table 3: Relationship between retention time, tailing factor, HETP, and number of theoretical plates of benzalkonium chloride HPLC and changes in mobile phase composition**

| Mobile phase composition | Retention time (min) | Tailing factor | HETP | Number of theoretical plates | Resolution |
|---------------------------|----------------------|----------------|------|-----------------------------|------------|
| Acetonitrile:water (75:25) | 9.351 | 0.946 | 0.0265 | 9404 | |
| Acetonitrile:water (60:40) | 27.701 | 0.999 | 0.0245 | 10163 | |

HETP: Height equivalent to a theoretical plate, HPLC: High-performance liquid chromatography

**Table 4: Relationship between retention time, the number of theoretical plates, the efficiency of the column, and a factor of benzalkonium chloride chromatogram on flow rate changes**

| Flow rate (mL/min) | Retention time (min) | Tailing factor | HETP | Number of theoretical plates | Resolution |
|-------------------|----------------------|----------------|------|-----------------------------|------------|
| 0.8               | 8.003                | 2.398          | 0.0219 | 4517 | 10.989 |
| 1.0               | 6.224                | 1.476          | 0.0254 | 2998 | 11.016 |
| 1.2               | 4.012                | 1.478          | 0.0245 | 7466 | 7.610  |

HETP: Height equivalent to a theoretical plate

**Table 5: Relationship between retention time, the number of theoretical plates, the efficiency of the column, and a factor of glutaraldehyde chromatogram on flow rate changes**

| Flow rate (mL/min) | Retention time (min) | Tailing factor | HETP | Number of theoretical plates | Resolution |
|-------------------|----------------------|----------------|------|-----------------------------|------------|
| 0.8               | 9.894                | 0.945          | 0.0219 | 11392 | 2.548 |
| 1.0               | 9.302                | 0.958          | 0.0254 | 9822  | 2.743 |
| 1.2               | 6.634                | 0.941          | 0.0245 | 10309 | 2.442 |

HETP: Height equivalent to a theoretical plate

**Table 6: Determination of benzalkonium chloride content of sample A**

| Sample | Concentration (ppm) | Peak area | Sample level (%) | Average sample level (%) | Levels in etiquette (%) | Recovery of etiquette (%) |
|--------|---------------------|-----------|------------------|--------------------------|-------------------------|---------------------------|
| A (benzalkonium chloride) | 100 | 49735 | 18.65 | 19.2 | 20 | 93.2 |
| B (benzalkonium chloride) | 100 | 51353 | 19.39 | 19.2 | 20 | 96.99 |
| C (glutaraldehyde content) | 100 | 51353 | 9.697 | 9.678 | 10 | 96.97 |
| D (glutaraldehyde content) | 12.5 | 11713876 | 29.04 | 29.82 | 30 | 100.13 |
| E (glutaraldehyde content) | D | 1528059 | 14.93 | 14.71 | 15 | 99.48 |
| F (glutaraldehyde content) | 1521606 | 14.5 | 14.5 | 15 | 96.8 |

Fig. 1: Benzalkonium chloride calibration curve. The curve was produced using 2.5, 5, 7.5, 10, 12.5, and 15 μg/ml benzalkonium chloride. The equation of the curve is y=255019x+220025 and the correlation coefficient (r) is 0.9995

Fig. 2: Glutaraldehyde chloride calibration curve. The curve was produced using 20, 50, 100, 200, 300, and 400 μg/ml glutaraldehyde. The equation of the curve is y=484.69x+4527.9 and the correlation coefficient (r) is 0.9995.
Method validation

Calibration curve
Glutaraldehyde and benzalkonium chloride standards of various concentrations produced the calibration curve through linear regression with correlation coefficients of 0.9995 (Figs. 1 and 2). Both of these correlation coefficients met the requirements (≥0.999).

Determination of LOD and LOQ
Assessment of the detection and quantitation limits is necessary to ascertain the lowest level at which a substance can be reliably detected and the lowest concentration at which the levels can be determined precisely, respectively. These are determined on the basis of statistical calculations through the linear regression of the obtained from the calibration curve. The LOD for benzalkonium chloride solution was 14.5 μg/mL and the LOQ was 48.5 μg/mL. In the case of glutaraldehyde, the LOD was 0.492 μg/mL and the LOQ was 1.640 μg/mL.

Selectivity
This parameter relates to the extent to which other substances may interfere with the method of analysis and are a measure of the method's ability to identify or measure the analyte in the presence of other substances from the sample matrix under predefined conditions. Specificity can be determined by adding ingredients that may be present in the sample [11].

Results from injection of 20 μL of glutaraldehyde in a placebo disinfectant matrix solution and also co-injection of DNPH showed no interference at the standard glutaraldehyde retention time. Likewise, no interference was found following an analysis of benzalkonium chloride in a placebo disinfectant matrix. This suggests that the method used is selective for glutaraldehyde and benzalkonium chloride compounds.

Accuracy and precision
Accuracy is the closeness between a test result and the true content of the analyte [12]. Accuracy should be performed in the same experiment using the same solution, and this parameter should be determined at three concentrations, including low, medium, and high [13]. Each level should be analyzed using three to six repetitions [14]. While good accuracy values range from 98% to 102%, for biological samples, this can range as widely as ±10%. Accuracy can be determined both absolutely and by addition [9].

This study used the simulation or spiked-placebo method to determine accuracy and precision. In the simulation method, standards are added to the placebo (disinfecting matrix) at various concentrations and then analyzed. Analytically, the determined levels are then compared with the known levels added to the placebo to obtain values for recovery (% R). At glutaraldehyde concentrations of 8, 10, and 12 μg/mL, recoveries were 99.04%, 99.41%, and 99.56%, respectively. For benzalkonium chloride at 80, 100, and 120 μg/mL, recoveries were 100.94%, 99.928%, and 99.974%, respectively. This result met the requirements of ≤2%.

Precision is generally measured as the coefficient of variation or standard RSD from the repeated analysis of freshly prepared quality control standards [11]. In general, a value of 2% or less is acceptable, but this value is flexible depending on the concentration of alkylate analyzed, the number of samples tested, and the conditions in the laboratory [2]. Precision is performed by calculating the RSD of triplicate analyses sample used for accuracy determination. The data obtained here met the requirements of ≤2%.

Determination of glutaraldehyde and benzalkonium chloride levels in disinfectant samples
Four samples consisting of two samples containing glutaraldehyde and two samples containing benzalkonium chloride were analyzed in duplicate, and the results are summarized in Tables 6

CONCLUSION
The HPLC analytical method for benzalkonium chloride and glutaraldehyde in disinfectants using UV-visible detection in this research was successful to produce a selective, accurate, and faster method.

CONFLICTS OF INTEREST
All authors have none to declare.

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