High performance liquid chromatography analytical method for glutaraldehyde determination in disinfectants

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Abstract

Glutaraldehyde (GA) is commonly used to disinfect surfaces and equipment in industries, agriculture, healthcare, laboratories, etc. To evaluate the quality of products containing GA, a HPLC method using 2,4-diphenylhydrazine derivative was developed. The analyte was separated by CN column with isocratic of acetonitrile and phosphoric acid 0,1% (ratio 50 : 50) and detected by PDA detector using a wavelength of 360 nm. The method was validated for specificity, linear curves, precision and accuracy which meet the requirements of AOAC.

Keywords: *Glutaraldehyde, glutaral, glutardehyde, aldehyde, DNPH, 2,4-diphenylhydrazine, disinfectant, HPLC.*

1. INTRODUCTION

Disinfectants are chemicals that can kill or inhibit the growth of harmful. They are diverse compounds with different chemical structures and uses, such as alcohols, chlorinated compounds, aldehyde compounds, hydroperoxides (hydrogen), peracetic acid, phenolic compounds, quaternary ammonium compounds, etc. Some substances can cause skin diseases when exposed such as chlorine, formaldehyde, and glutaraldehyde, so users should be provided with adequate information about the chemicals being used to choose appropriate products and use them effectively.

Glutaraldehyde (GA) is a saturated dialdehyde (Figure 1) with a high degree of bactericidal activity, commonly used as a 2% solution to disinfect medical devices. GA has most of the characteristics: rapid action, broad antibacterial spectrum, good cleaning ability, lasting effect on the surfaces of treated instruments [1]; unaffected by environmental factors, odorless or pleasant, capable of dilution, has stable concentrations even when diluted, toxicity is not too severe [2], economical, easy to use. In practice, 0.1 - 2% glutaraldehyde

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solutions are often used for sterilization or for immobilization of samples in the cytological analysis under the microscope. A solution with a concentration of 10 - 15% is used in water treatment, controlling the growth of bacteria, algae, fungi, and viruses in the aquatic environment. Some commercial products of 50% glutaraldehyde which are considered non-hazardous to the environment are used to kill bacteria and fungi to help eliminate the cause of disease in farmed shrimp.

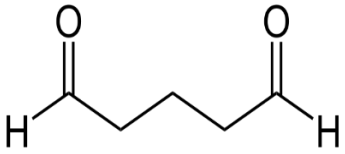

Figure 1. *Chemical structure of glutaraldehyde*

Many methods were developed and applied for determination of GA content. The most commonly used methods are titration [3-4] and colorimetry [5]. These methods are simple, easy to implement, but not selective for specific aldehydes. More specific methods are capillary electrophoresis (CE) [6], high performance liquid chromatography (HPLC) using UV-Vis/PDA detector [7-8], and gas chromatography (GC) with flame ionization detector (FID) [9], electron capture detector (ECD) [10] or mass spectrometry detector (MS) [11].

Currently, in Vietnam, there is no official method to quantify GA by HPLC in disinfectants. Therefore, a high-performance liquid chromatographic method with PDA detector through derivatization with characteristic yellow color with 2,4-dinitrophenylhydrazine (DNPH) was chosen to conduct the study to determine the GA content because it is a stable method, easy to deploy, suitable for laboratory conditions. The reaction between GA and DNPH is depicted in Figure 2

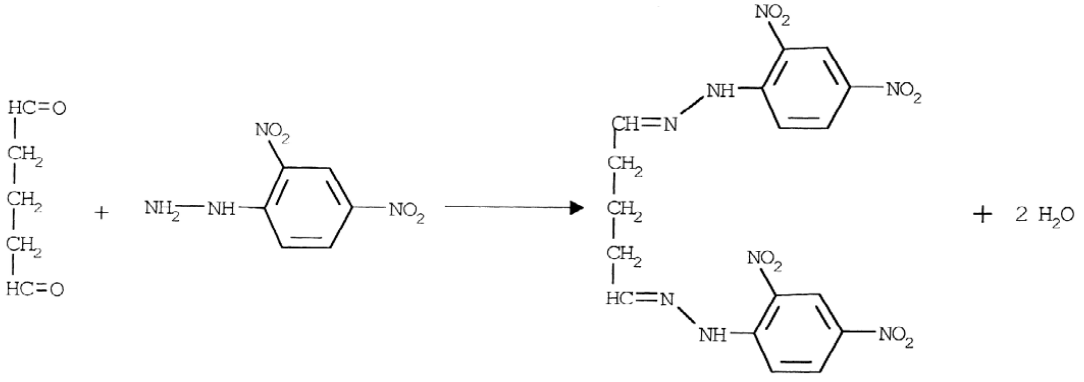

Figure 2. *Reaction between Glutaraldehyde and DNPH*

According to the EPA method 8315A of the US Environmental Protection Agency [12], the free carbonyl compounds were determined by derivatization with the reagent 2,4-dinitrophenylhydrazine (DNPH) at pH 3 to produce hydrazone, then analyzed by HPLC with UV-Vis detector at 360 nm. This is the basis to conduct a number of surveys to optimize the method.

2. MATERIALS AND METHODS

2.1. Chemicals and equipment

2.1.1. Equipment

The main equipment used in this study was the Alliance high-performance liquid chromatography system with a Waters PDA detector, an Eclipse XDB-CN chromatographic column (250 mm × 4.6 mm, 5 µm, Agilent), a Symmetry C18 column (250 × 4.6 mm, 5 µm, Waters), and some other common equipment and tools in the laboratory.

2.1.2. Chemicals

The chemicals used in the study were analytical purity, including 25% Glutaraldehyde solution (Sigma Aldrich), reagent 2,4-dinitrophenylhydrazine (DNPH, China), acetonitrile (Merck), phosphoric acid 85% (Merck), purified water for chromatography.

2.2. Objective

The research object is GA-containing disinfectant products at National Institute for Food Control.

2.3. Method

2.3.1. Investigation of chromatographic conditions for analysis of glutaraldehyde by HPLC

Analytical chromatographic separation of the GA 50 g/mL standard solution using an Eclipse XDB-CN chromatographic column (250 × 4.6 mm, 5 µm) (Agilent) with a mobile phase system consisting of acetonitrile and 0.1% phosphoric acid with different rate. Standard solutions were derivatized with 2.5 µg/mL DNPH reagent in ACN containing 1% H_3PO_4 (v/v) in a 1 : 1 ratio and analyzed by HPLC.

2.3.2. Investigate the derivation conditions of GA with DNPH

GA was determined by derivatization with DNPH reagent to give a colored hydrazone product and determined at 360 nm. In order for GA to react completely, it is necessary to ensure that the DNPH reagent is always in excess of the amount of GA in the analytical sample.

To optimize this process, a number of experiments are carried out to make the reaction quick and simple:

- Investigation of phosphoric acid concentration and reaction time
- Monitoring the stability of the derivative product over time
- Investigation of reaction temperature.

2.3.3. Method validation

The method after being developed was validated according to the criteria required by AOAC including: specificity, calibration curve, precision and recovery on the matrix of disinfectant preparations.

3. RESULTS AND DISCUSSIONS

3.1. Resuls of investigation of chromatographic conditions

By reference to the previous studies [7, 11] and based on our laboratory conditions, Symmetry C18 columns (250 mm × 4.6 mm, 5 µm) (Waters) and Agilent Eclipse XDB-CN columns (250 mm × 4.6 mm, 5 µm) were selected to investigate the chromatographic separation conditions. The GA-DNPH 50 µg/mL standard solution was analyzed isotropic with a mobile phase consisting of ACN:H_3PO_4 0.1% in the ratio 60 : 40, detected at 360 nm. The chromatogram was shown in Figure 3.

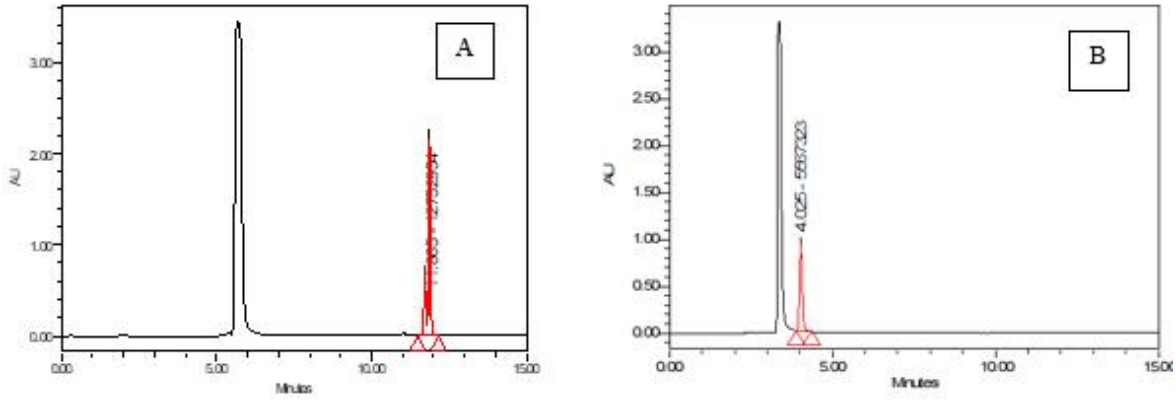

Figure 3. *Chromatogram of 50 µg/mL GA standard solution after derivatization: A: C18 column; B: CN column*

Analytical chromatograms of standard solutions of GA-DNPH using column C18 (Figure 3A) showed good retention. However, two peaks appeared on the chromatograms, which are geometric isomers of EE, EZ-hydrazone. The analysis result on the CN column (Figure 3B) showed that only one peak appears, the retention time is short, but the analyte peak has not been completely separated from the reagent peak. Therefore, column CN was selected for further study of mobile phase ratio ACN - H_3PO_4 0.1% in the condition of 60: 40, 55: 45, 50: 50. The results show that the ratio of 50: 50 gave good separation of the analyte peak and the reagent peak (Figure 4).

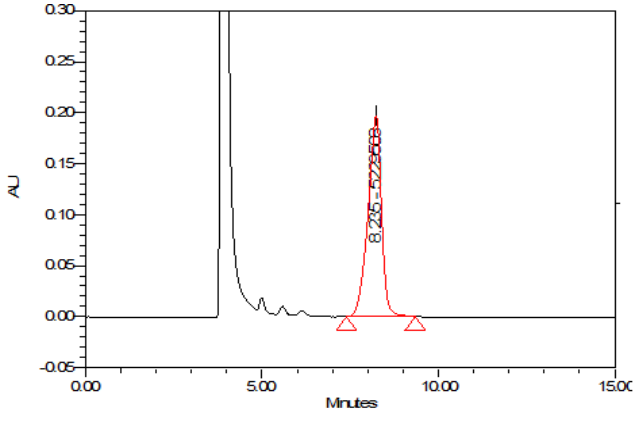

Figure 4. *Chromatogram of GA standard solution 50 µg/mL after derivatization under selected conditions: Eclipse XDB-CN column (250 mm × 4.6 mm, 5 µm), mobile phase: ACN: H_3PO_4 0.1% (50 : 50)*

3.2. Investigate the derivation conditions of GA with DNPH

Samples containing GA are often combined with some components such as quaternary ammonium compounds (alkyl dimethyl benzyl ammonium chloride, dialkyl dimethyl ammonium chloride, etc.), ethanol to form a liquid preparation... These substances do not affect the sample preparation, therefore, investigate the derivatization conditions of GA using DNPH reagent. According to Menet [7], linearity is ensured when the ratio of DNPH: GA ≥ 32 even at high concentrations (310 - 2,500 µg/mL). Thus, in order to simplify the derivatization process, and ensure that the reagents are always in excess, the GA sample was diluted, the DNPH reagent at a concentration of 2,500 µg/mL or 2.5 mg/mL was used to perform the reaction at the ratio of 1 : 1.

3.2.1. Investigation of phosphoric acid concentration and reaction time

Through reference to the literature, the GA 50 µg/mL reaction experiment with 2.5 mg/mL DNPH reagent was performed in an ACN containing H_3PO_4 at concentrations of 0.01, 0.1, and 1%. After the reaction time of 15, 30, 45 and 150 min, the samples were analyzed. The results were shown in Figure 5.

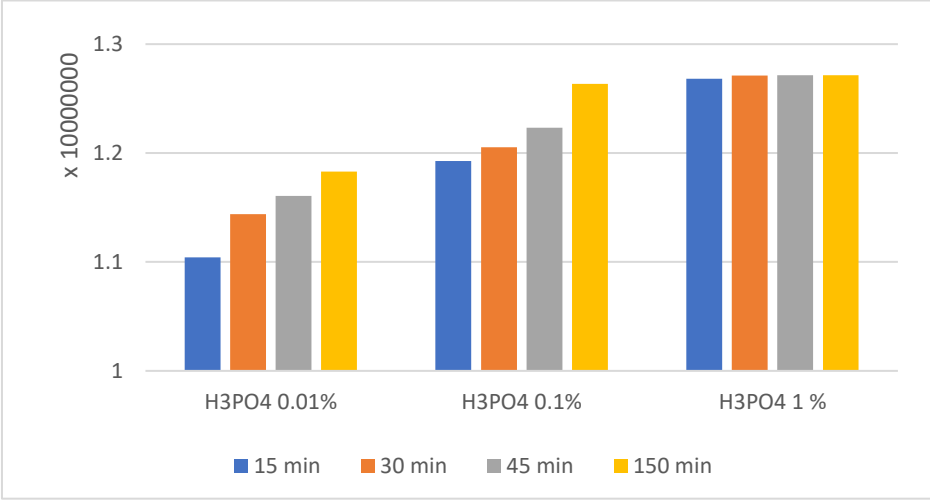

Figure 5. *Results on the effect of time and phosphoric acid concentration*

The results in Figure 5 showed that the GA-derivative peak area using the DNPH reagent containing 0.01 and 0.1% phosphoric acid is always smaller than the peak area using the reagent containing 1% acid concentration at different reaction times. That means the derivatization reaction may not take place completely. The results of data analysis (ANOVA-one way) using Microsoft Excel software showed statistical differences when using different acid concentrations, with p = 0.0003.

Comparison of the derivatization times for 15, 30, 45, and 150 min showed that at 1% acid concentration, the peak area did not change significantly with time, indicating that the reaction took place completely in 15 min at phosphoric acid concentration of 1%. This result is consistent when using the analysis of variance, the p = 0.4639 value shows that the different results are not statistically significant. DNPH 2.5 mg/mL solution in ACN containing 1% phosphoric acid and reaction time of 15 min was selected for further investigations.

3.2.2. Monitoring the stability of the derivative product over time

Temperature is a factor that can affect the rate and efficiency of a derivatization reaction. According to Menet [7], GA at low concentrations (1.25 - 10 µg/mL) has a coefficient R^2 of the standard curve which can be lower than 0.999 if not heated. Therefore, the effect of temperature on reaction performance is considered over the expected concentration range of calibration curve (1 - 10 µg/mL). Carry out a derivatization reaction of GA standard solution with concentration in the range of 1 - 10 µg/mL with DNPH 2.5 mg/mL reagent at room temperature and 45°C. The results were shown in Table 1.

Table 1. *Investigated results of the temperature at 45°C (A) and room temperature*

C (µg/mL)	***Area (mAU.s)***		***Retention time (min)***	
	A	***B***	***A***	***B***
10.0	1057368	1082152	9.014	9.018
5.00	526710	524846	9.023	9.039
2.00	209235	201670	9.026	9.048
1.00	67437	96126	9.028	9.062
Calibration curve	y = 108567x - 23362 R^2 = 0.9994	y = 109666x - 17299 R^2 = 0.9999		

Using Microsoft Excel software for single-factor analysis of the peak area and retention time of hydrazone derivatives obtained in Table 1, the results show that the peak area and retention time at two temperatures are similar ($p < 0.05$), showing that the yield and rate of the derivatization reaction are not affected by temperature. The calibration curve for heated derivatization had a coefficient R^2 no better than without heating (Table 1), contrary to initial expectations. Therefore, GA derivatization can be carried out at various concentrations with DNPH reagent in ACN containing 1% H_3PO_4 at room temperature to simplify sample preparation.

Thus, after the survey, the GA analysis procedure was selected as follows: dilute 1 mL of the test sample to 50 mL with water. If the sample has a high concentration, it can be diluted appropriately before analysis. Conduct derivatization between sample or standard

solution and 2.5 mg/mL DNPH reagent in ACN containing 1% phosphoric acid in 1: 1 ratio for 15 min, then analyze by HPLC-PDA. Chromatographic conditions using Agilent Eclipse XDB-CN column (250 mm × 4.6 mm, 5 µm), with mobile phase including ACN : phosphoric acid 0.1% (ratio 50 : 50, v/v), flow rate 1.0 mL/min, detection wavelength 360 nm.

3.3. Validation of developped method

3.3.1. Specification

Proceed to derivatize the blank sample, 50 µg/mL standard solution, test sample with DNPH reagent, and then analyze on HPLC device. The obtained results showed that the retention time of the GA derivative in the standard addition sample is the same as that in the standard sample (8.90 min). On the blank sample chromatogram, there is no peak in the retention time of the analyte (Figure 6).

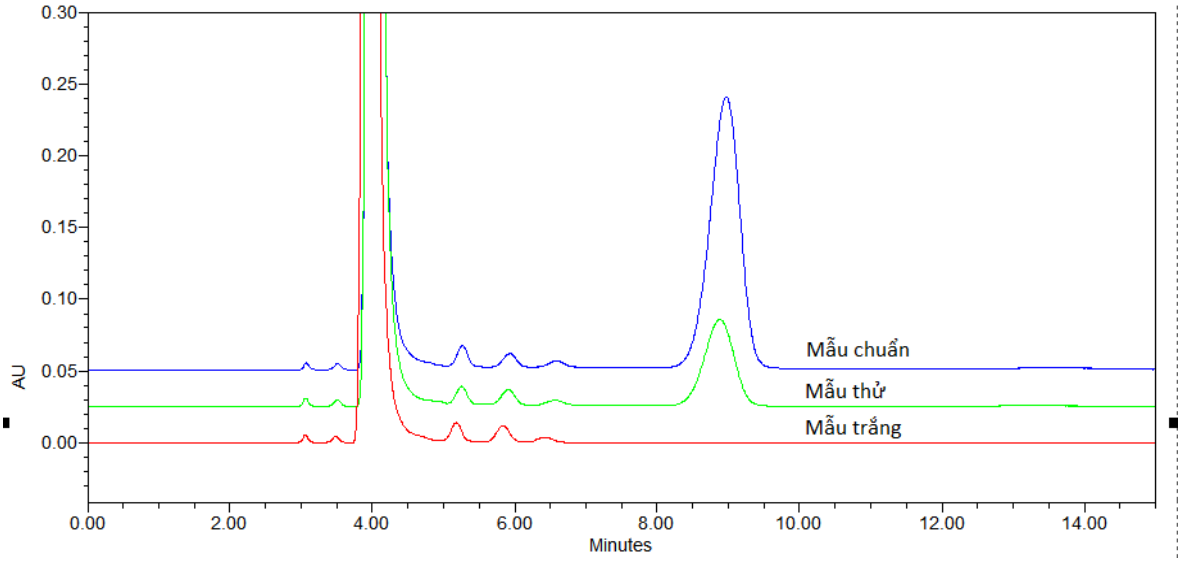

Figure 6. *Chromatographic of standard, blank and test samples*

3.3.2. Calibration curve

Analyze standard solutions with concentrations in the range of 5 - 50 µg/mL and establish the calibration curve. It was found that GA concentration and peak area were linear over the concentration range 5 - 50 µg/mL (Figure 7) with coefficient $R^2 \geq 0.995$ with bias at all points ≤ 15%.

With the linear range of themcalibration curve, the method is suitable for the analysis of GA-containing preparations, usually with concentrations in the range of 0.1 - 25%, equivalent to 1,000 - 25,000 µg/mL.

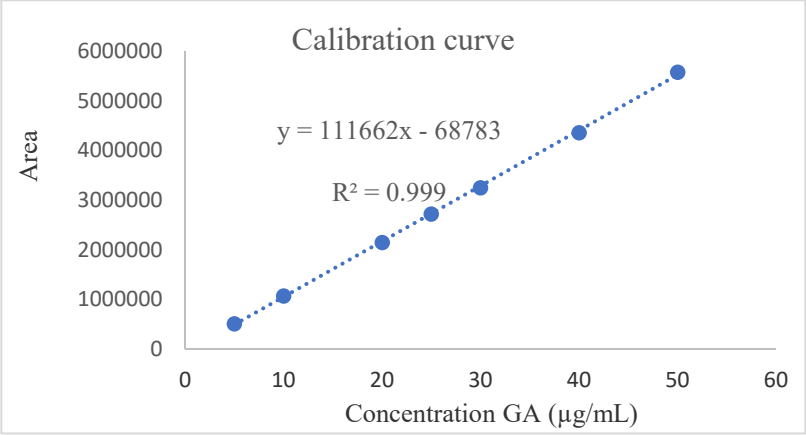

Figure 7. *Calibration curve of GA*

3.3.3. Precision and accuracy

Precision is determined through repeatability and reproducibility. Analyze a sample 6 separate times for repeatability and analyze another 6 times at another interval to determine reproducibility (intermediate precision). The repeatability and reproducibility results are summarized in Table 2.

Accuracy: The correctness of the method is determined through the recovery. Carry out the standard addition to the real sample at three concentrations of 2.50, 12.5 and 25.0%, each level is performed 04 times, analyze and calculate the recovery efficiency. The recovery results are summarized in Table 2.

Table 2. *The results of the analysis of precision and recovery*

Parameter	***Concentration***	***Results***	***AOAC requirements***	***Conclusion***
Repeatability	16,4%	1,08%	≤ 1,3%	Meet the requirements
Reproducibility		1,37%	≤ 1,7%	
Recovery	2,50%	97,2 - 100%	97 - 103%	
	12,5%	97,8 - 101%	97 - 103%	
	25,0%	98,0 - 102%	98 - 102%	

From the validation results, it can be seen that the developed method meets the AOAC requirements for repeatability, reproducibility and recovery, and the method can be applied for GA analysis.

4. CONCLUSION

The study investigated and built a simple method to analyze GA in disinfectant preparations by derivatization with DNPH reagent, then analyzed on HPLC equipment using

PDA detector, detection and quantification at a wavelength of 360 nm. The method was validated for specificity, calibration, precision and accuracy. The results show that the method meets the requirements according to AOAC, which can be applied to analyze GA content in disinfectant preparations. This method can be developed to simultaneously analyze several other aldehydes in disinfectant preparations such as formaldehyde, glyoxal, methylglyoxal, etc., which is also the next research direction of the research team.

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Phương pháp sắc ký lỏng hiệu năng cao xác định hàm lượng glutaraldehyd trong chế phẩm khử khuẩn

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Tóm tắt

Glutaraldehyd (GA) là một hoạt chất được sử dụng phổ biến để khử trùng và sát khuẩn bề mặt và thiết bị trong công nghiệp, nông nghiệp, y tế, phòng thí nghiệm,... Nhằm đánh giá chất lượng các chế phẩm chứa GA trên thị trường, phương pháp xác định GA bằng cách dẫn xuất với thuốc thử 2,4-dinitrophenylhydrazin (DNPH) đã được xây dựng. Chất phân tích được tách sắc ký bằng cột CN với pha động đẳng dòng gồm acetonitrile và acid phosphoric 0,1% (tỷ lệ 50 : 50) và xác định bằng detector PDA ở bước sóng 360 nm. Phương pháp đã được thẩm định độ đặc hiệu, đường chuẩn, độ đúng và độ chụm đạt yêu cầu của AOAC.

Từ khóa: *Glutaraldehyd, glutaral, aldehyd, DNPH (2,4-dinitrophenylhydrazin), khử khuẩn, khử trùng, HPLC.*