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Fast specific separation and sensitive quantification of bactericidal and sporicidal aldehydes by high-performance liquid chromatography: example of glutaraldehyde determination

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Abstract

This article describes the design and the validation of the HPLC determination of glutaraldehyde at g/l and mg/l concentrations, after derivatization by 2,4-dinitrophenylhydrazine and using the external standard method. At low concentrations, the reaction mixture needs to be heated and a weight ratio of 500 for the 2,4-dinitrophenylhydrazine reagent and the glutaraldehyde ensures a linear calibration curve. In contrast, high concentrations do not require heating of the reaction mixture and a weight ratio of 32 proved to be sufficient. The optimized HPLC method has been validated for both ranges of concentrations. Between 1.25 and 10 mg/l, the content can be determined by the external standard method, with a repeatability of 0.5%. The detection limit is 0.2 mg/l. Between 0.31 and 2.5 g/l, the content can also be determined by the external standard method, with a repeatability of 0.4%. Finally, statistical analysis has demonstrated that aqueous solutions of glutaraldehyde are stable for at least three days at 4°C within the mg to g range.

Keywords: Glutaraldehyde; Aldehydes

1. Introduction

Aldehydes are molecules known as disinfectants: glutaraldehyde, formaldehyde and glyoxal are contained in many cleaning and disinfectant products for medical and surgical equipment and of surfaces (e.g., Pyobactene, Detercide, etc.). The Pitié-Salpétrière Hospital Center uses 2% (w/w) aqueous solutions of glutaraldehyde, either as is, e.g., Cidex (2%, w/w in glutaraldehyde), or diluted in water, e.g., Korsolex (38.5%, w/w in glutaraldehyde). In particular, these solutions are used for soaking bathes to disinfect endoscopes according to a procedure which consists of cleaning of the endoscopes with a detergent before soaking in a glutaraldehyde solution and a final rinse with sterile water.

Such a use of glutaraldehyde has raised two problems, namely, the stability of glutaraldehyde in the soaking bathes and its toxicity, as the final rinsing may not completely remove it from medical and surgical equipment.

However, no data are available in the literature on the stability of glutaraldehyde. It is nevertheless known that glutaraldehyde forms polymers in alkaline, neutral or acidic solutions [1]. The polymerization reaction is partly reversible. Its stability

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can also depend on substances that were not completely eliminated by a preliminary cleaning of the medical and surgical equipment. So far it is recommended to keep commercial aqueous solutions at a concentration of 25% (w/w) at 4°C and protected from light.

Finally, residual glutaraldehyde may cause necrosis as has been observed after endoscopic examination [2]. A close monitoring of the residual quantity on the walls of the endoscope is consequently required.

To carry out these two studies, it is necessary to design a determination method for the various disinfectant aldehydes, in particular for glutaraldehyde. The method must be product specific, linear in the g/l and mg/l ranges of concentrations, and should have a limit of detection sufficiently low to detect glutaraldehyde traces on the equipment after final rinsing.

A few methods for detection and quantification of aldehydes have already been described in the literature. They are based on volumetric methods (iodometry [3] for instance), colorimetric methods (by reaction with amino compounds [4] for example) and chromatographic methods (gas chromatography, GC [5] and high-performance liquid chromatography, HPLC [6–10]). The first two methods can not be used to quantitate specific aldehydes, unlike the chromatographic methods. Moreover, for some given colorimetric methods, sugars and proteins possibly contained in soaking baths, interfere with the quantification of the product [11]. Lyman et al. [5] quantified glutaraldehyde by GC with an internal standard, using high concentrations (from 1 to 6 g/l), but no detection limit was given. In the same way, HPLC was already used for the quantification of aldehydes after their derivatization with 2,4-dinitrophenylhydrazine (DNPH) [6–10]. However the derivatization conditions were not optimized for low concentrations.

We have therefore decided to compare the GC and HPLC detection limits in a preliminary study to check if they are in the same range as the tolerated concentration limits, ca. 1 ppm in the atmosphere [1], i.e. ca. 1 mg/l in solution. It led us to design an HPLC method based on the external standard method after derivatization of the glutaraldehyde with DNPH. Finally we have analyzed our results with the help of statistical tools in terms of accuracy, precision and linearity [12] of the method and in terms of solution stability.

2. Experimental

2.1. Reagents

For both GC and HPLC methods, a commercial solution of glutaraldehyde [25% (w/w) in water] (Merck-Clevenot, Nogent sur Marne, France) was directly used as a standard. It was stored at 4°C and protected from light.

2.1.1. Specific reagents for GC

The GC internal standard, i.e. 2-(2-ethoxyethoxy)-ethanol [same as di(ethylene glycol) monoethyl ether], was obtained from Sigma (St. Quentin Fallavier, France), methanol from Merck (HPLC-grade quality for chromatography).

2.1.2. Specific reagents for HPLC

DNPH was obtained from Sigma and recrystallized from ethanol [9]. Hydrochloric acid (35.5% HCl) was RP Normapur quality and purchased from Prolabo (Paris, France).

2.2. Apparatus

2.2.1. Gas chromatography

A Girdel 75 equipped with a flame ionization detector and a recorder Kipp and Zonen Model BD 111 (Touzart et Matignon, Les Ulis, France) was used. The column was a 5 m×1/8 in. (1 in.=2.54 cm) inox tube packed with 15% carbowax 20M–2% KOH on Chromosorb W AW 60–80 mesh (Touzart et Matignon). The operating parameters were a 125°C temperature for the column, 200°C for the outlet and the injector and a 45 ml/min flow-rate for the nitrogen carrier gas.

2.2.2. Liquid chromatography

The HPLC system consisted of an isocratic pump (Spectra-Physics, Model 8800, Thermo Separation Products, Les Ulis, France) and a Rheodyne valve 7010 fitted with a 10-μl injection loop. A UV absorbance detector (Spectra-Physics, Model UV

100, Thermo Separation Products) was used at a 358 nm wavelength and at a sensitivity of 0.5. The analytical column was a Nucleosil C_{18} RP (250×4.6 mm I.D., 5 μm particle size, 100 Å pore diameter) (AIT, St Germain en Laye, France). Data were computed on an integrator (Spectra-Physics, Model 4400, Thermo Separation Products) with attenuations equal to 32 and 256 for low and high concentrations, respectively.

Mobile phases for HPLC were made of various mixtures of acetonitrile (HPLC-grade quality LiChrosolv for chromatography, Merck) and distilled water (Pharmacie Centrale des Hôpitaux, Paris, France).

2.3. Preparation of standards

2.3.1. Preparation of GC standards [5]

The standards were used to measure the detection limit obtained with a flame ionization detector (FID) with GC. The commercial glutaraldehyde solution (25%, w/w) was diluted to various levels. The diluted solutions were introduced into 10-ml flasks. A constant volume (5 ml) of a 10 g/l methanolic solution of 2-(2-ethoxyethoxy)ethanol (internal standard) was added into each flask. Methanol was then added to fill the 10-ml flask.

We have injected 2-μl aliquots of each standard and have determined the peak-height ratio of glutaraldehyde (first eluted) to internal standard for each injection.

2.3.2. Preparation of HPLC standards and samples

Before the make up of standards, the DNPH reagent was prepared by adding 0.2 g of DNPH to 100 ml of ACN at room temperature followed by stirring for 0.5 h. The hydrochloric acid solution was prepared by diluting 10 ml of 35.5% HCl solution to 10 ml of water.

High concentrations. Before use, the commercial glutaraldehyde solution (25%, w/w) was diluted with water to obtain a 2.5 g/l concentration. This standard glutaraldehyde solution (2.5 g/l) and the samples were diluted 20 times with distilled water. Aliquots of 0.25, 0.5, 1 and 2 ml of this glutaraldehyde reference standard solution and 2 ml of the sample were pipetted into separate 10-ml volumetric flasks. A volume of 4 ml of the DNPH reagent and 400 ml of hydrochloric acid were added before diluting to volume with distilled water.

Low concentrations. The standard solution (2.5 g/l) was diluted 250 times with distilled water. Aliquots 0.25, 0.5, 1 and 2 ml of this glutaraldehyde standard solution and 2 ml of the sample were pipetted into separate 10-ml volumetric flasks. Volumes of 5 ml of the DNPH reagent and 400 μl of hydrochloric solution were added before diluting to volume with distilled water.

2.3.3. Injection

Aliquots of 10 μl of each solution and sample were directly injected in the column using a manual injection valve. Glutaraldehyde peak area was then plotted against the glutaraldehyde concentration (0.3125, 0.625, 1.25 and 2.5 g/l for high concentrations and 1.25, 2.5, 5 and 10 mg/l for low concentrations).

For the g/l sample, an additional dilution was in some cases necessary. Indeed, the crystals which may appear originated from an intermediate derivative.

3. Results and discussion

The derivatization of aldehydes such as glutaraldehyde to their corresponding 2,4-dinitrophenylhydrazone, named "DNPHones" is shown in Fig. 1.

The resulting hydrazone has an absorbance maximum at 358 nm.

3.1. Optimization of chromatographic conditions

To achieve an accurate and reliable determination of glutaraldehyde, the retention time of DNPHone should be significantly different from the hold-up time to prevent possible interferences caused by an excess quantity of reagent which elutes close to this hold-up time. Mobile phases for HPLC consisted of various mixtures of acetonitrile and distilled water; its flow-rate was always set at 1.5 ml/min. Fig. 2

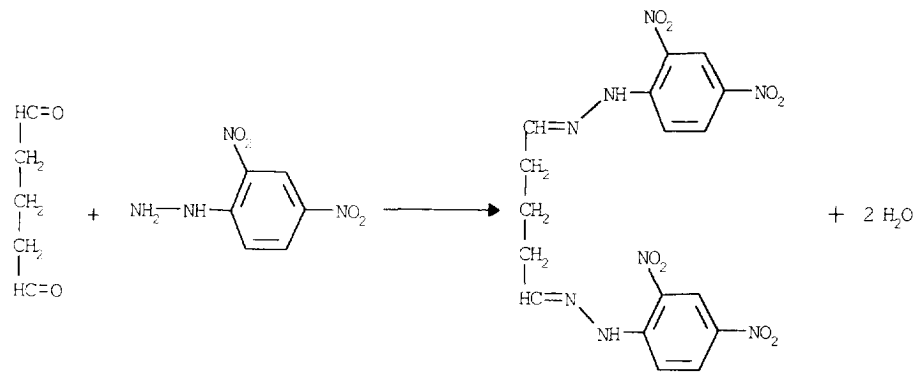

Fig. 1. Derivatization of glutaraldehyde to its corresponding 2,4-dinitrophenylhydrazone.

shows the retention time variation of DNPHone and of the main peak of the reagent versus the acetonitrile–water ratio. The latter was finally chosen as 55:45 (v/v), the DNPHone retention time is indeed smaller than 15 min and sufficient compared to the 2.8 min retention time of the reagent.

Fig. 3 shows chromatograms of a non-heated and a heated reagent. Degradation peaks appear after the heating required for low glutaraldehyde concentrations. Fig. 4 shows chromatograms of low concentration of glutaraldehyde and demonstrates that degradation peaks of the reagent do not interfere with the DNPHone. Fig. 5 shows the chromatogram of high concentration of glutaraldehyde.

Preliminary experiments carried out before any statistical studies show that the relative standard deviation calculated for 20 injections was satisfactory only if the chromatographic column is cleaned after each analysis with acetonitrile as was recommended by Risner et al. [8]. A small increase of the peak area is indeed observed if no rinsing is done between two experiments; such a phenomenon could originate from a memory effect of our HPLC apparatus. As it is not reasonable to rinse after each

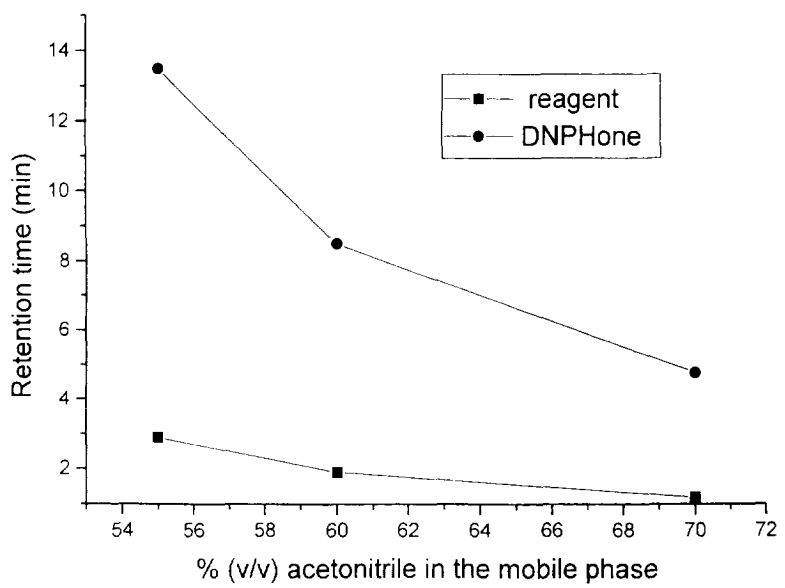

Fig. 2. Retention times of "DNPHone" and main peak of the reagent versus the % (v/v) acetonitrile in the mobile phase.

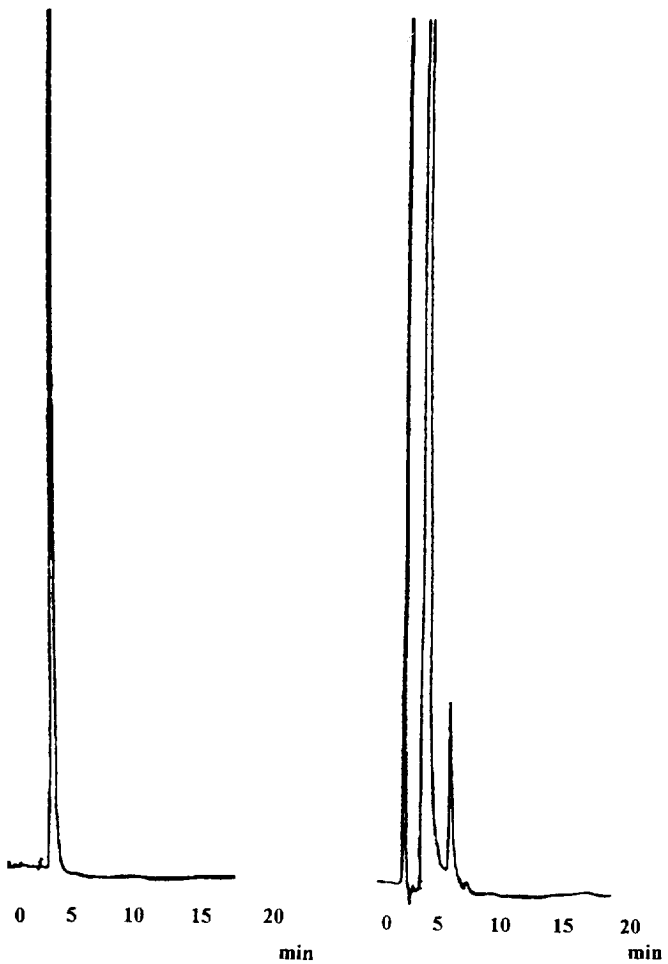

Fig. 3. Chromatograms of a non-heated (left) and a heated (right) reagent.

experiment, successive analyses were done to determine the number of injections in series which leads to less than 2% relative standard deviation: it was concluded that 10 was satisfactory.

3.2. Glutaraldehyde derivatization

We have designed the running conditions (heating temperature and duration, quantity of the derivatization reagent) according to the calibration curves at ranges from 0.31 g/l to 2.5 g/l and from 1.25 to 10 mg/l. In order to quickly define the derivatization conditions and as the HPLC method should show a linear response, the correlation coefficient for each standard curve was used as a discriminating number. A value higher than 0.999 validates the derivatization conditions used.

3.2.1. High concentrations

First, we have tested the need to heat the mixture of glutaraldehyde and DNPH reagent to obtain a quantitative derivatization as recommended by Demko et al. [7]. We have plotted calibration curves with no heating and with a mass ratio of DNPH to glutaraldehyde set at 32; the stoichiometric ratio is equal to: 188 divided by 100 (DNPH molecular mass on glutaraldehyde molecular mass). Correlation coefficients are always higher than 0.999. Further experiments have shown that a ratio of 4 was not satisfactory (correlation coefficient <0.999) in con-

Fig. 4. Chromatogram of "DNPHone" glutaraldehyde at a concentration of 10 mg/l.

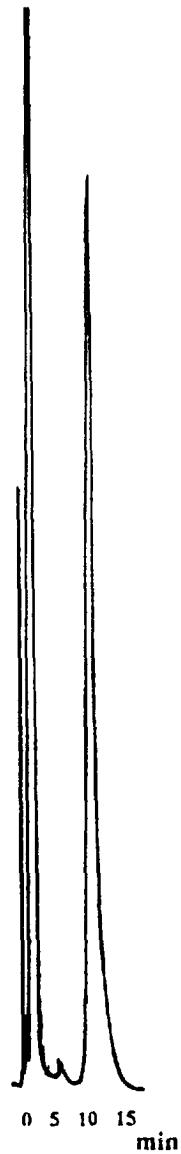

Fig. 5. Chromatogram of "DNPHone" glutaraldehyde at a concentration of 2.5 g/l.

trast to a ratio of 32. Subsequently, we did not heat the derivatization mixtures for high glutaraldehyde concentrations and we used a 32 mass ratio.

3.2.2. Low concentrations

First, we have tested the need to heat the reaction mixture including a large excess of the DNPH reagent (mass in DNPH/mass in glutaraldehyde= 500). Correlation coefficients versus reaction time are always lower than 0.999 after 24 h with no heating. We have consequently decided to heat at 45°C, as indicated by Demko et al. [7]. A reaction time of 45 min with a 45°C temperature ensures a linear (r>0.999) response. A time shorter than 40 min only ensures the linearity between 2.5 and 10 mg/l of glutaraldehyde. A heating time longer than 50 min leads to a lack in linearity probably related to solvent evaporation.

Secondly, we have examined the quantity of DNPH which was necessary to obtain a linear calibration curve. Several experiments were carried out with a DNPH mass/glutaraldehyde mass ratio

from 50 to 500. Ratios of 250 and 500 met our specifications on r, but a 500 ratio was chosen to improve the ruggedness of the method.

Thereafter, we have heated the reagent mixtures at 45°C during 45 min, with a DNPH mass excess of 500, for the determination of low glutaraldehyde concentrations.

3.3. Statistical studies

Analytical validation of the quantification methods described for either high or low concentrations of glutaraldehyde is based on the study of precision, linearity and accuracy. The detection limit was determined for low glutaraldehyde concentrations, and compared to that obtained by GC.

3.3.1. HPLC versus GC: detection limit

For HPLC and GC methods, the background noise (BN) was evaluated by injecting blank solutions and by measuring the higher amplitude of the signal along a distance equal to twenty times the half-height width of the glutaraldehyde peak [12]. The detection limit (DL) was then evaluated by: DL=3×BN. The HPLC method allows to detect a quantity of glutaraldehyde twenty five times smaller than the GC method and gives a detection limit of 2 ng (corresponding to an injection of 10 μl of a 0.20 mg/l solution) against 50 ng in GC (corresponding to an injection of 2 μl of a 25 mg/l solution). Moreover, this quantity detected in HPLC may be easily decreased by increasing the injection volume.

This consequently confirmed our choice of an HPLC method to quantify glutaraldehyde.

3.3.2. Precision

The precision encompasses different parameters, i.e. repeatability and reproducibility.

Repeatability. The repeatability takes into account the smallest variability of the methods for glutaraldehyde determination. It was estimated by the analysis of six successive injections (n=6) of a single 2.5 g/l solution and six successive injections (n=6) of single 10 mg/l solution. The injections were performed on the same day using the same reagent, the same technician and the same apparatus. Relative standard deviations are satisfactory at 0.5% and 0.4% for high and low concentrations, respectively.

Reproducibility. Two factors, i.e. day of analysis and the solution preparation, are studied by analyzing three different solutions (named a, b and c) injected one time on each of three days (day 1, day 2 and day 3), (n=9). Between each day, the solutions were stored at 4°C.

Relative standard deviations are satisfactory at 0.75% and 2.5%, respectively for high (2.5 g/l) and low (10 mg/l) concentrations.

3.3.3. Linearity

Estimation of parameters for the regression line. The study was carried out during three days. Each day, the calibration curve was plotted with four points, each representing a single injection. The four solutions containing different glutaraldehyde concentrations were prepared the first day from a stock solution at 2.5 g/l stored at 4°C. The linearity has been studied for both ranges of concentrations: from 0.31 to 2.5 g/l ($y=-177+20307x$, y in arbitrary units, r=0.9992, n=12) and from 1.25 mg/l to 10 mg/l ($y=-174+293x$, y in arbitrary units, r=0.9990, n=12).

y intercept and zero. A zero intercept is required for a standardization method based on a single point (it requires a direct proportion between the concentration and the peak area). This test consists in the comparison between the value of the y intercept and 0 by a Student test on the ratio of the intercept on its standard deviation. The discriminating value at a 95% confidence interval is t 10,95%=2.228 (10 degrees of freedom).

For high concentrations the standard line intercept is not significantly different from zero at 95% confidence interval (t=177/380=0.476<2.228), in contrast to low concentrations (t=174/23=7.565> 2.228).

A standard line is therefore required for low concentration determination.

3.3.4. Accuracy

Examples of confidence intervals are calculated using averages and variance results obtained during repeatability: 2.50±0.01 g/l and 10.01±0.04 mg/l.

3.4. Stability study

Reproducibility results have allowed the calculation of solution concentrations stored at 4°C during three days, the concentrations on the first day taken as 100% for simplification purposes. For the second day, the values are 98%, 102% and 103% for the low concentrations and 102%, 100% and 102% for the high ones. For the third day, the values are 97%, 104% and 96% for the low concentrations and 103%, 100% and 102% for the high ones. An analysis of variance was carried out with two controlled factors (day and solution preparation) in order to evaluate the stability of the glutaraldehyde solutions stored for three days at 4°C. Formulas used are described by Fleury [12]. Estimated variances related to the solution preparation (S_p^2, 350 391 and 152 for the high and the low concentrations, respectively) and to the day (S_d^2, 512 931 and 547 for the high and the low concentrations, respectively) have been compared to the residual variance (S_r^2, 50 171 and 1333 for the high and the low concentrations, respectively) using the Fisher–Snedecor test ($F(2,2,5\%)=19$). The value is always higher than the ratio S_p^2/S_r^2 and $>S_d^2/S_r^2$, for both concentrations.

The day and preparation factors do not influence the response. Therefore the solutions of glutaraldehyde (2.5 g/l and 10 mg/l) are stable at least for three days at 4°C.

4. Conclusion

This paper has shown that a reliable HPLC method was designed for glutaraldehyde determination in the mg/l and g/l concentration ranges. The derivatization of glutaraldehyde by DNPH is achieved when the reagent is 32 times in excess in weight compared to glutaraldehyde in the g/l range with no heating or 500 times in excess in the mg/l range, requiring a 45-min heating at 45°C. These conditions ensure the linearity of the method, its repeatability (lower than 1%) and its reproducibility (smaller than 2%). Moreover, solutions are stable over 3 days.

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