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A GC-FID validated method for detection and quantification of ethylene oxide in urine bags

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ABSTRACT

The urine bag is a thermo-labile medical-hospital device used in clinical/surgical procedures in urine drainage and collection. As it is a thermolabile material, it is commonly sterilized by ethylene oxide, a highly toxic, mutagenic and carcinogenic cyclic ether gas. Currently, several countries require, through documents and standards, the quantification of ethylene oxide in medical and hospital devices. Thus, this work describes the development and validation of a method employed for the quantification of ethylene oxide (ETO) in urine bags. The method was developed based on the guidelines of ISO 10993-7, ANVISA and INMETRO. The work range, linearity, limits of detection and quantification, repeatability, intermediate precision and selectivity were evaluated. Statistical tools such as Levene's test, Grubbs, residual analysis, F-test of two variances, paired sample T test and ANOVA table, were also applied to evaluate the method. The linearity of the work range (10 – 400 $mg.L^{-1}$) showed an adequate correlation coefficient ($r > 0.9993$), with a homoscedastic profile with absence of outliers while the limit of detection and quantification were 1,95 and 6,5 $mg.L^{-1}$ respectively. After validation, 45 samples of urine bags of different batches were evaluated, which demonstrated levels of ETO below the limit of detection (1,95 $mg.L^{-1}$). The results highlight a simple method that meets several regulations with a wide working range, high sensitivity and capability to quantify ETO not only in urine bags but also in other medical devices.

Keywords: ethylene oxide, GC-FID, urine bags, medical devices.

1. INTRODUCTION

Ethylene oxide (EtO) is a cyclic ether gas with a three-membered ring which was discovered by Wurtz around 1859 [1, 2]. However, it was later employed only in the year 1962 as a sterilization agent for thermosensitive and/or hydrosensitive medical and hospital materials such dialysis membranes, catheters, infusion sets, urine bags, and others [3-5]. Its action is based on microorganisms destruction during sterilization because of its capability to penetrate the cell, reach the DNA, and interacting chemically with the proteins essential for cell reproduction by alkylation [6-9]. Ethylene oxide is also known its toxicity, mutagenicity, carcinogenicity and hence can cause irreversible damage to health if an individual is exposed to it [5, 10-12].

The risks behind the use of EtO is related to its presence in medical devices after the sterilization process. During the sterilization EtO can be entrapped in polymeric constituents according to the variation in the time that the material is submitted to the aeration step which can cause a toxic effect when the polymer comes into contact with living tissue or even blood [2, 7, 13, 14]. Studies indicate that plastic tubes sterilized by EtO can cause hemolysis when in contact with human blood [2, 13]. Some manuscripts also highlight the ability of EtO to remain in several unclosed polymeric materials at various temperatures above its boiling point [13, 15]. It is also known that the residue of EtO in medical and hospital materials can irritate the tis-

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sues of patients when they get into contact with the patients' mucous membranes and can also trigger mutagenesis or carcinogenesis [2, 4, 8, 11]. In some cases, the residue of EtO has been known to cause allergic reactions [3, 8, 16, 17].

In view of the risks related to the use of EtO, guidelines were created to control its use. In Brazil the use of EtO as a sterilizing agent is regulated by the Ministries of Health and Labour through the Interministerial Ordinance no. 482, April 16, 1999 [18]. This ordinance indicates the health and occupational safety conditions to be contemplated by sterilization companies, as well as the permitted residual quantities of this substance in medical and hospital materials after sterilization. The maximum residual value of EtO in medical devices or materials of different types have been regulated to be between 5 and 250 $mg.L^{-1}$ depending on the type of material, where these limits are also determined by the ISO Standard 10993-7 [19].

There is, therefore, the need to evaluate all medical and hospital devices that undergo sterilization with this substance, employing reliable analytical methods, and to validate the analytical procedure according to guidelines stipulated by regulatory organs such as the ANVISA (National Health Surveillance Agency Brazil), the INMETRO (National Institute of Metrology, Standardization and Industrial Quality, Brazil), FDA (Food and Drugs Administration, United States of America) and others. It is interesting to note that these guidelines can be updated from time to time by the regulatory organs in order to improve on analytical data and results, like ANVISA and INMETRO that recently updated their guidelines of validation of analytical methods [20, 21].

Gas chromatography (GC) is an analytical technique widely employed for the analysis of EtO and has been coupled to various sampling techniques as well as different types of detectors. HS-SPME-GC/FID, SPME-GC/FID, HS-GC/MS, GC/ECD, GC/FID, and HS/GC/FID are some of the hyphenated techniques that have been used to evaluate EtO [2, 7, 22-29]. All these previous methods provide good results with a very low detection limit (detection at ppm and ppb levels) but they are expensive, require costly sample preparation steps, and were constructed with a short work range to determine EtO in some polymeric materials and specific medical devices. These limitations do not allow them to have a wide working range and a cost-benefit analysis required to determine EtO in all medical devices according to the actual guidelines.

Thus, taking into account the toxicity of ethylene oxide, the importance of the quantification of its residues in medical and hospital materials, and the limitations presented by present analytical methods, we hereby expose the evaluation of ethylene oxide in medical urine drainage bags using a newly developed, sensitive and validated GC-FID analytical method based on the recently updated guidelines of the ANVISA and INMETRO [20, 21].

2. MATERIAL AND METHODS

2.1 Reagents

The standard of ethylene oxide (EtO) in acetone (10,100 $mg.L^{-1}$, 100%) was purchased from Solutech (São Paulo, SP, Brazil). Ultrapure water (resistivity of 18,2 MΩ.cm) was obtained from a Master System All MS2000 - Gehaka system (São Paulo, SP, Brazil).

2.2 Preparation of solutions and standards

A stock solution of EtO (500 $mg.L^{-1}$) was prepared by adding 2,475 mL of the standard to 47,525 mL of water employing calibrated micropipettes. It was stored at -5 °C and discarded after 5 days.

2.3 Description of samples

Forty-five samples of urine bags from different batches were donated by Medical (Pernambuco, Brazil) for EtO residual analysis. All samples were given codes, stored at ambient temperature and evaluated following the procedures described below.

2.4 Preparation of samples

The samples were prepared according to ISO 10993-12: Sample Preparation and Reference Materials with modifications [30]. From each sample, with the help of a pair of scissors, three pieces of 6 cm^2 were cut, shredded and transferred into 20 mL vials containing 2 mL of deionized water. The vials were sealed hermetically and samples were incubated for 5 hours at 37 °C. Aliquots of extracts of 1 mL were filtered (0,2 µm syringe filters) into 2 mL vials and submitted for analysis.

2.5 GC-FID system and chromatographic conditions

The analytical method was developed with a Clarus 680 Gas Cromatograph Perkin Elmer (Waltham, Massachusetts, USA) consisting of a furnace for column, split/splitless capillary injector and automatic injector for 108 samples with a flame ionization detector (FID). An Elite-Wax column (30m x 0,25mm x 0,25µm, column with its phase consisting of polyethylene glycol) was used with the following analytical parameters: oven with an initial temperature of 50 °C for one minute, with a ramp rate of 18,0 °C per minute and final temperature of 200 °C. The analysis time was 9,33 minutes, and helium was used as the mobile phase with a flow rate of 0,8 $mL.min^{-1}$. Injection volume was 1 µL at an injection temperature of 230 °C, with a split ratio 10:1. The temperature of the FID detector was set at 220 °C with hydrogen and synthetic air flow rates of 40 $mL.min^{-1}$ and 400 $mL.min^{-1}$, respectively.

2.6 Method Validation

The working range and linearity, limit of detection (LD), limit of quantification (LQ), precision expressed as repeatability and intermediate precision, accuracy and matrix effect were evaluated. The validation procedures followed the criteria of the updated documents, CGCRE-008, Rev. 07, July 2018, issued by the National Institute of Metrology, Quality and Technology [20] and the Resolution No. 166 of the National Agency of Sanitary Surveillance [21].

2.7 Selectivity - Matrix Effect

For the evaluation of selectivity, it was necessary to prepare a matrix solution according to ISO 10993:12 with some modifications [30]. Briefly, pieces of free-ethylene oxide (unsterilized) urine bags were incubated in water, in hermetically sealed vials for five hours, in an oven at a constant temperature of 37 °C. The extract was filtered to obtain the matrix solution.

The matrix effect was evaluated by comparing replicates with three concentration levels of EtO (10, 200 and 400 $mg.L^{-1}$), one prepared in water (wat) and the other in matrix solution (mat), as described above. The F (Snedecor) and T (student) tests were used to evaluate the results to verify if the matrix had an effect on the precision and concentration of the analyte.

2.8 Working range and linearity

Calibration curves of the analyses of ethylene oxide (in triplicate) in different concentrations were elaborated (10, 50, 100, 200, 300, 400 $mg.L^{-1}$). The absence of outliers for each level of concentration was verified with the Grubbs test. Homoscedasticity (equality of variances) was verified by the Levene´s test, while the simple linear regression was evaluated by the unweighted ordinary least square method. The random behaviour of the residuals was evaluated graphically, and linearity by the F-test.

2.9 Limit of Detection and Quantification

The limit of detection was determined based on the signal-to-noise ratio of 3:1, where the analyte (EtO) signals were compared with those of blank samples (water), establishing the minimum concentration at which EtO can be detected. Likewise, the limit of quantification was determined by comparing measured analyte signals to those of blank samples and establishing the concentration at which the analytes can be reliably quantified with a signal-to-noise ratio of 10:1.

2.10 Repeatability, intermediate precision and accuracy

Six replicates at the minimum, medium and maximum levels of the working range (10, 200 and 400 $mg.L^{-1}$) were analysed in order to evaluate the accuracy, and three replicates of the entire working range (10, 50, 100, 200, 300 and 400 $mg.L^{-1}$) prepared on different days by different analysts were used for intermediate precision assay. To determine repeatability three replicates were studied at the same levels used in the intermediate precision study. To assess whether the groups tested (different analysts) were considered statistically similar, the results of studies carried out under varying conditions were compared using the F and T tests. Repeatability and intermediate precision were expressed as relative standard deviation (% RSD). An acceptance criterion for accuracy was between 80 and 120%.

2.11 Robustness

Robustness was evaluated by varying the injection port temperature and the flow rate of the carrier gas, as proposed in the Resolution No. 166 of the National Agency of Sanitary Surveillance [21]. The variation lev-

els (aprox. ± 5% of the original analytical parameter) used for this study are presented in table 6, where three replicates at the concentration of 10 mg.L^{-1} were studied for their accuracy and relative standard deviations.

3. RESULTS AND DISCUSSION

3.1 Working range and linearity

The linearity of the method was evaluated by triplicates of analytical curves of EtO standard prepared in the working range (10-400 mg.L^{-1}). The calibration curves obtained by the least squares regression analysis showed excellent correlation coefficients (r> 0.9993), as observed in the mean curve (Figure 1S, Supporting Information). The guidelines of the ANVISA [21] stipulates that the correlation coefficient should be above 0.99. However, the new guidelines of the INMETRO [20] does not make mention of an exact correlation coefficient (even though the old guideline stipulates it to be above 0.90) but it requires the analysis of outliers for each concentration level and homoscedasticity [31]. The curves were homoscedastic according to the Levene´s test with the absence of outliers - Grubbs test [32] and with a random residual plot (supplementary information). The F values calculated by ANOVA (table 1) were higher than the table value and p-value was less than 0.05, showing a statistically valid linear regression and demonstrating that the linear model adequately described the correlations between peak areas and concentrations in the evaluated working range as described in the work of Cheibub and colleagues [33]. The regression equation of the average curve was used for quantification.

Table 1: Analysis of variance (ANOVA) of the least squares linear regression model.

	D.F.	SUM OF SQUARES	AVERAGE SQUARES	F. TAB.	F. CALC.	P-VALUE
Curve	5	346234,263	69246,8526	3,106	693,6413413	2,45332E^{-14}
Residuals	12	1197,971029	99,83091906			

D.F.: Degrees of Freedom.

3.2 Limit of detection and limit of quantification

The limit of detection (LD) of EtO was determined at 1,95 mg.L^{-1} and the limit of quantification (LQ) was 6.50 mg.L^{-1}, values lower than 25 mg.L^{-1} maximum limit of ethylene oxide that may be present in correlates that might have contact with blood, as in the case of the urine bag. This indicates that our proposed method is sufficiently sensitive and robust for the analysis of EtO residues in hospital and medical materials with the exception of intrauterine biomaterials whose maximum residual limit should be 5 mg.L^{-1} [18]. Notwithstanding, Dias et al., developed a method by GC-FID to evaluate EtO in oxygenators and tubings applied to the heart and demonstrated the LD and LQ to be 0,42 mg.mL^{-1} and 1,38 mg.mL^{-1}, values which are lower than the ones indicated in this work [34]. However, contrary to our work, they did not show the working range and linearity of their method.

3.3 Repeatability, intermediate precision and accuracy

The accuracy of the EtO in samples varied between 96,4 and 119,1% as shown in table 3. The repeatability was between 3,86 and 6,42%, while the intermediate precision was less than 10% (table 2). F test values (p> 0,05) showed that the concentration values obtained individually by each analyst were statistically equal. The T test (p <0,05) showed that the concentration values measured by different analysts and on different days are statistically equal. All values met the criteria defined by INMETRO and ANVISA, with the exception of the accuracy value of the minimum concentration (10 mg.L^{-1}), 119,1 %, which was higher than that stipulated by the INMETRO. However, the RDC 166 of the ANVISA does not stipulate the accepted accuracy value [20, 21]. These results confirm the repeatability and reproducibility of the method.

Table 2: Accuracy, repeatability (studied at three levels of concentration) and intermediate precision given in relative standard deviation (RSD %).

ANALYTE CONCENTRATION ($mg.L^{-1}$)	ACCURACY (%)	REPEATABILITY (RSD %)	INTERMEDIATE PRECISION (RSD %)		TEST F		TEST T	
			ANALYST 1	ANALYST 2	ANALYST 1	ANALYST 2	DAY 1	DAY 2
10	119,11	6,42	7,51	7,80	0,0732	0,2972	0,0034	0,0002
50	-	-	3,00	10,01				
100	-	-	6,56	3,92				
200	96,35	3,86	2,96	11,07				
300	-	-	4,91	5,83				
400	100,70	4,92	7,80	2,10				

RSD: Relative Standard Deviation. Test F ($p>0,05$). Test T ($p<0,05$).

3.4 Selectivity - Matrix Effect

Matrices of samples may possess components which may interfere in the detection and adequate quantification of samples. This can cause a bias by increasing or decreasing the signals of the analytes and changing the slope of the calibration curves [20, 35]. According to the INMETRO, the matrix effect can be evaluated by preparing two groups of test samples, one with the matrix solution and the other with a solvent [20]. Both of the test sample groups should have the same analyte concentration at the same level of the concentration of interest, evaluating the low, medium and high levels, with at least 3 replicates for each level of concentration. In this work, the matrix effect was evaluated by comparing two replicates of three concentration levels (10, 200 and 400 $mg.L^{-1}$), one prepared in water (H_2O) and the other in matrix solution (Mat), as described above in the methods section. The concentration response and precision for each concentration level were evaluated by the F-Snedecor test and later by the t-test. The concentration response of each level was not affected by the matrix according to the F (Snedecor) distribution and the t (student) test results ($p <0,05$) as shown in table 3.

Table 3: Results of Selectivity - matrix effect F Snedecor test and t-test.

ETO CONCENTRATION IN WATER (H_2O) AND MATRIX (MAT) SOLUTION	TESTE F SNEDECOR	TESTE T STUDENT
C10 (H_2O) – C10 (Mat)	0,1729	0,4656
C200 (H_2O) – C200 (Mat)	0,0705	0,1913
C400 (H_2O) – C400 (Mat)	0,8835	0,5210

3.5 Robustness

The variations in the injection port temperature and carrier gas flow rate did not significantly affect the accuracy results for the concentration at 10 $mg.L^{-1}$. According to the RDC 166 of the ANVISA with regards to robustness in the case of quantitative methods, the impact of the proposed variations on the obtained results should be evaluated with the same criteria used for the accuracy [21]. However, this validation parameter is not obligatory for the INMETRO. In this study, the variations of the carrier gas flow rate gave accuracies of 101% and 119% (Table 4) which is similar to the accuracy (119,1) obtained from the original carrier gas flow rate (Table 2). However, the RSD´s (%) of the accuracy of the variations of the carrier gas flow rate were higher (16,9% and 11,8% respectively; Table 6) than the RSD (%) of the accuracy of the original gas flow rate (6,4%; Table 3). The variations of the injection port temperature in relation to the original injection port temperature produced an accuracy of 91,5 % and 89,2 %, which are in consonance with the accuracy range stipulated by the INMETRO at this concentration [20]. Table 4 presents the results found for robustness.

Table 4: Variables and variation levels evaluated and results of Robustness.

VALIDATION PARAMETERS FOR ETO AT 10 mg.L^{-1}	EQUIPMENT OPERATING CONDITIONS			
	CARRIER GAS FLOW RATE (mL.min^{-1})		INJECTION PORT TEMPERATURE (° C)	
	0,76 (-5%)	0,84 (+5%)	220 (-5%)	240 (+5%)
Accuracy	118,9	101	89,2	91,5
Precision (%RSD)	11,8	16,9	7,7	3,2

RSD: Relative Standard Deviation.

3.6 Samples Analysis

Urinary bags are used together with catheters in the management and monitoring of a patient's condition. They can be used to empty the contents of the bladder, e.g. before or after abdominal, pelvic or rectal surgery and before certain investigations. They are used to determine residual urine, to relieve retention of urine and to measure urine output accurately [36-38]. The need to sterilize these products with ethylene oxide is important and more so, the evaluation of the residual content of this chemical or sterilizing agent is of great importance, since if it is not below the maximal residual limit, the products may cause irritation or toxicity.

Forty-five samples of urine bags from different batches were analyzed and evaluated using this present developed method. The amount of EtO present in the urine bags was lower than the LD (1,95 mg.L^{-1}), which is lower than 25 mg.L^{-1}, the maximum concentration limit allowed for EtO in urine bags.

4. CONCLUSION

Chromatographic methods used to quantify EtO are expensive, complex, with low robustness, and quantify EtO in a restricted range of medical devices. In this way, for the first time, a simple, robust, and sensitive GC-FID method was successfully developed, validated, and implemented for the determination of EtO in biomaterials based on the guidelines of ISO 10993-7, ANVISA and INMETRO. The work range, linearity, limits of detection and quantification, repeatability, intermediate precision and selectivity were applied to evaluate the method.

The linearity of the work range (10 – 400 mg.L^{-1}) showed an adequate correlation coefficient ($r > 0.9993$) with an homoscedastic profile and with absence of outliers while the limit of detection and quantification were 1,95 and 6,5 mg.L^{-1}, respectively. The accuracy varied between 96,4 and 119,1% and the intermediate precision was less than 10% with T test ($p < 0,05$) was statistically equal by different analysts and on different days. Variations in the injection port temperature and carrier flow rate did not significantly affect the method accuracy. These results confirm the repeatability, reproducibility, and robustness of the method. After validation, the method was used to evaluate EtO in 45 urine bags from different batches, where the concentration of EtO was found to be below LD (1,95 mg.L^{-1}).

Future work should also include the optimization of the method for quantifying simultaneously ethylene chlorohydrin (ECH) and ethylene glycol (EG), two subproducts of EtO that can be present in medical devices and are controlled by ISO 10993-7.

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