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Original Article

Validation of Free Formaldehyde Determination Method in DT Vaccine and Tetanus Toxoid Antigen

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Abstract

Validation is a Good Manufacturing Practice principle that proves any procedure, process, method, equipment, material, activity, or system actually leads to the expected results. This study validates the method for the determination of free formaldehyde in biological products (including the diphtheria-tetanus vaccine and tetanus toxoid antigen). The operating procedure of this method is based on pharmacopoeial monographs. It also does not require full validation, although its suitability under the actual condition of use should be verified. Performance characterizations, such as accuracy, intra-precision (repeatability), intermediate precision (inter-precision), linearity, range, and the limit of quantitation, were investigated and calculated. Accuracy and precision were studied at different concentration levels by spiking known amounts of formaldehyde in real samples. The accuracy and precision results were expressed as the recovery and the relative standard deviation (RSD), respectively. Precision was expressed as intra-precision (repeatability) and inter-precision. Intra-precision or repeatability was performed by one operator in one day by adding three levels of concentration to the products. The inter-precision was conducted by one operator in three individual days within the same laboratory at three concentration levels. Range and linearity were assessed by investigating the correlation coefficient of the regression line between different concentrations of formaldehyde and their response. The acceptance criteria and limits were defined for these validation parameters in these biological products. The RSD for intra-day and inter-day precision studies was less than 5% in a medium concentration of linear range. At this concentration level, accuracy was 90%-110%. The method's linearity ranged between 0.0000039%-0.01% w/v of formaldehyde with a correlation coefficient of 0.9999. The results exhibited sufficient linearity, accuracy, precision, and range. Therefore, this method can be used successfully to determine free formaldehyde for biological products.

Keywords: Accuracy, Linearity, Precision, Repeatability, Verification

1. Introduction

Formaldehyde has a long and extensive history of use in the preparation of bacterial and viral vaccines for inactivation (1). Free formaldehyde residuals can remain in the final vaccine product despite several manufacturing steps followed in the inactivation process. Therefore, it is important to monitor the residual levels of formaldehyde in the product to evaluate manufacturing consistency (2). The requirements for using formaldehyde and the permitted residual amount of formaldehyde allowed for human bacterial vaccines and the diphtheria-tetanus antigen have been specified by the World Health Organization as requirements for diphtheria, tetanus, pertussis, and combined vaccines (3). Monitoring programs should be performed to limit

and control the potential health risk of this probable carcinogenic compound. There are also different methods for formaldehyde determination (4). British Pharmacopoeia (5) has explained a spectrophotometry method for determining free formaldehyde in vaccines. In this method (lutidine method), the Hantzsch reaction is used to derivatize formaldehyde by cyclizing acetylacetone and formaldehyde in the presence of ammonia to 3,5-diacetyl-1,4-dihydrolutidine (6). This method is described in British Pharmacopoeia as a monograph. Although users of compendial analytical procedures are not required to validate these procedures (7), it is necessary to verify this method's suitability under actual use conditions for its application in different vaccines with a different matrix. In other words, according to the Good Manufacturing Practice principles, the validity and proper performance of the method must be proven under laboratory conditions (8). In this method, validation and verification include assessing the elements, such as the effect of the matrix on the recovery of formaldehyde extraction from the vaccine matrix. Validation of an analytical procedure is the process by which laboratory studies are used to establish that the performance characteristics of the procedure meet the requirements for its intended use (9). Typical analytical performance characteristics that should be considered in the validation of methods were described in different guidelines (7, 10-12). The present study investigated the validation of the free formaldehyde determination method in diphtheria-tetanus (DT) vaccines and tetanus toxoid antigens. Compendial assay procedures vary from highly exacting analytical determinations to the subjective evaluation of attributes. Considering this variety of assays, it is logical that different test methods require different validation schemes, and the validation parameters of each method must be defined separately depending on the type of method. The United States Pharmacopoeia (USP) (9) and the International Council for Harmonisation (ICH) (11) divide the types of common compendial methods into four main categories. Table 1 shows different elements required for validation in each category (9). The table assigns a specific parameter required for validation for each category. For the free formaldehyde determination method, performance characteristics, including accuracy, precision, linearity, range, and the limit of quantitation (LOQ), were investigated and calculated.

The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range (9). The range of an analytical method is the interval between the upper and lower levels of the analyte that should be determined with a suitable level of precision, accuracy, and linearity using that method (9). The LOQ is the lowest concentration of an analyte in a sample that may be determined with acceptable accuracy and precision. Accuracy is the closeness of test results obtained by that method to the true values (10). The ICH recommends (11) accuracy assessments using a minimum of nine determinations over three concentration levels, covering the specified range. Specificity is defined as the ability to assess the analyte unequivocally in the presence of components expected to be present, such as impurities (11). In an analytical procedure for impurities, specificity may be established by spiking the product with appropriate levels of impurities and demonstrating that these impurities are determined with reasonable accuracy and precision (7, 9). Precision is the degree of closeness between individual test results when a method is applied repeatedly to multiple samplings of a homogeneous sample (9) The ICH guidelines (11) define three levels of precision when applied to an analytical procedure which should be established during method validation. In this study, the acceptance criteria and limits were defined for these validation parameters in DT vaccines and tetanus toxoid antigens. The results were evaluated, analyzed, and compared against the pre-determined acceptance criteria.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals and Samples

Anhydride acetic acid, formaldehyde (37% w/v), and ammonium acetate were purchased from Merck (Darmstadt, Germany), and acetylacetone was purchased from BDH (Sydney, Australia). The DT vaccines and tetanus toxoid antigen were provided by the Human Bacterial Vaccines Department of Razi Vaccine and Serum Research Institute (Karaj, Iran).

2.2. Apparatus

The reagent was heated in a Huber Polystat CC2 water bath. Ultrapure water was prepared by an Ultra Clear TWF EI-Ion (SG, Germany). The UV-Visible Spectrophotometer Ultrospec 3000 was used for the absorbance measuring of solutions.

2.3. Analytical Procedure

The derivatization agent was prepared by dissolving 7.5 gr of ammonium acetate, 0.15 mL of glacial acetic acid, and 0.1 mL of acetylacetone in pure water and diluting the mixture to 50 mL using pure water (5). In a test tube, 1.0 mL of formaldehyde sample (or standard solutions), 4.0 mL of pure water, and 5.0 mL of acetylacetone reagent (derivatization agent) were subsequently added. The final solution was heated at 40°C using a circulating water bath and allowed to stand for 40 min. The absorbance of the solution was measured at 640 nm. A solution containing 5.0 mL of derivatization agent and 5.0 mL of water was used as the blank solution. The content of formaldehyde in the vaccine and antigen to be examined was calculated from the established calibration curve using the standards (reference) solutions. The method procedure was conducted as described in Pharmacopoeia (5); however, it was necessary to make changes in the sample preparation method in the laboratory. Due to the very low concentration of formaldehyde in the DT vaccine, the vaccine was used instead of pure water in the sample preparation. Therefore, 5 mL of the derivatization agent and 5 mL of the vaccine sample were combined to prepare a sample solution. Moreover, because of the similarity of the color of the tetanus toxoid antigen with the color of the derivatization agent solution, 0.5 mL of the antigen sample, 4.5 mL of pure water, and 5.0 mL of the derivatization agent were combined to prepare a sample solution. A solution consisting of 0.5 mL of the antigen sample and 9.5 mL of water was used as the blank solution.

Finally, the residual formaldehyde concentration in the sample was calculated according to the sample volume and dilution factor.

2.4. Method Validation

According to the USP categorization (9) shown in table 1, the free formaldehyde determination method is used to determine residual impurities in a quantitative assay. This method falls in Category II of this classification. Therefore, accuracy, precision, specificity, LOQ, linearity, and linear range are parameters normally required to validate the free formaldehyde determination method. The following describes how to evaluate each parameter separately.

2.5. Linearity

In this study, the linearity of the method was detected by preparing five formaldehyde concentrations (0.000157, 0.000314, 0.000627, 0.001254, and 0.0025% w/v). They were then taken for the assay as described in the procedure. The lowest concentration was selected according to the permitted residual amount of formaldehyde in human bacterial vaccines and the amount of formaldehyde in these products. The dilution factor was considered in the sample preparation. Subsequent concentrations were chosen to be twice the previous concentration.

Linearity between the absorbance and formaldehyde concentrations was evaluated by calculating the regression line using the least squares method. Therefore, the test data were subjected to statistical analysis to calculate the regression line's correlation coefficient, y-intercept, and slope.

Table 1. Data elements required for validation (9)

Analytical Performance Characteristics	Category I	Category II		Category III	Category IV
		Quantitative	Limit Test		
Accuracy	Yes	Yes	*	*	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	*	Yes
Detection Limit	No	No	Yes	*	No
Quantitation Limit	No	Yes	No	*	No
Linearity	Yes	Yes	No	*	No
Range	Yes	Yes	*	*	No

* May be required, depending on the nature of the specific test

2.6. Range and Limit of Quantitation

The range of the method was investigated by preparing different concentrations of formaldehyde solution, and their responses were determined. The correlation coefficient of the regression line between these concentrations and their response was then calculated. Afterward, the lowest and highest formaldehyde concentrations decreased and increased, respectively, to the extent that the determined response did not show the appropriate value of linearity or accuracy and precision. The distance between the highest and lowest concentration levels of formaldehyde was reported as a range, which showed good linearity with the response to the other concentration levels. In addition, the lowest calculated concentration was reported as the LOQ.

2.7. Accuracy and Specificity

In this study, accuracy was assessed in biological products spiked with known amounts of formaldehyde in three concentration levels by five replicates of each concentration.

The permitted residual amount of formaldehyde in human bacterial vaccines and tetanus antigens is less than 0.02% w/v. In addition, the amount of formaldehyde in tetanus toxoid antigen is about 0.002% and even less in DT vaccines. According to these values and the linearity range of the method, formaldehyde was spiked in three concentration levels of 0.01%, 0.002%, and 0.0005% w/v to biological products, and the amount of formaldehyde added was determined by the mentioned method. In calculations, the concentration of spiked formaldehyde was computed by subtracting the initial formaldehyde concentration in the product as the blank solution.

Specificity was evaluated similarly to accuracy by spiking with a known level of formaldehyde in products, and then the amount of spiked formaldehyde was determined. In fact, the specificity parameter studies were performed with precision, and the accuracy parameter studies were conducted together.

2.8. Precision

Precision is usually expressed in terms of intra-precision (repeatability) and intermediate precision (inter-precision). Repeatability refers to the use of an analytical procedure within a laboratory over a short period using the same analyst with the same equipment. Inter-precision is defined as laboratory variation on different days or with different analysts or equipment within the same laboratory. In this study, both types of precision were expressed as the relative standard deviation (RSD) of a series of measurements at three concentration levels. One operator performed an intra-precision study or repeatability in one day by adding three levels of concentration to the products. One operator performed an inter-precision study in three days within the same laboratory by adding three levels of concentration to the products.

3. Results

3.1. Linearity

Five solutions were prepared with different concentrations of formaldehyde, and their formaldehyde content was measured by the method (Table 2). The calibration curve was obtained by the linear regression of the absorbance of the solution versus concentration. The obtained regression equation was Y=266.77X+0.0017, where Y is the absorbance of the formaldehyde solution, and X is the free formaldehyde concentration in % w/v. The coefficient of determination (R^2) of 0.9999 (n=5) proved excellent linearity between the response (absorbance) and free formaldehyde concentration.

Table 2. Concentration of formaldehyde solutions and their absorbance

(X_i) Formaldehyde concentration (% w/v)	(Y_i) Absorbance of solution
0.000157	0.043
0.000314	0.088
0.000627	0.169
0.001254	0.333
0.0025	0.67

Slope=266.77
Y-Intercept=0.0017
Correlation coefficient (R^2)=0.9999

A high R^2 of 0.99 is often used as the criterion of linearity (13). Accordingly, the free formaldehyde determination method showed excellent linearity in biological products (Figure 1).

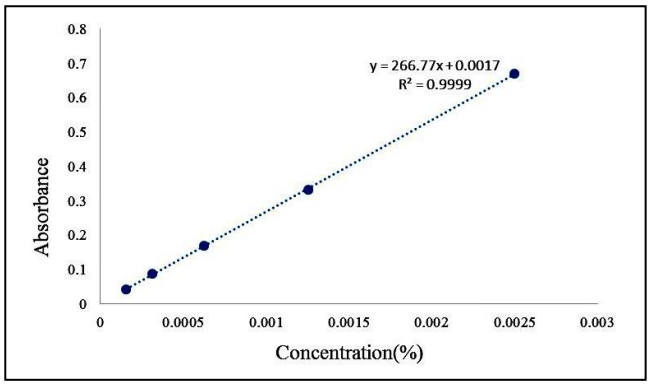

Figure 1. Linearity of free formaldehyde determination method

3.2. Range and Limit of Quantitation

The range for the free formaldehyde determination method was calculated based on the distance between the highest and the lowest concentration of formaldehyde. The results showed good linearity with the response of other concentrations (0.0000039%-0.01% w/v of formaldehyde). For formaldehyde concentrations of more than 0.01%, the absorbance measured by the spectrophotometer is more than about 2.5. According to the spectrophotometer model and absorbance uncertainty issues (9, 14), an absorbance of greater than 2.5 in this spectrophotometer has more uncertainty. As a result, if the amount of formaldehyde in the sample is more than 0.01% w/v, the sample must be diluted first with pure water, and if the amount is less than 0.0000039% w/v, it should be used instead of pure water in the sample preparation. For example, the amount of formaldehyde in the DT vaccine is usually much lower than its specification. Therefore, in the sample preparation, as mentioned in the analysis method, the vaccine is used instead of pure water to increase the amount of formaldehyde in the final sample. Therefore, this method can properly measure a sample with a calculated amount of formaldehyde as more or less than the range by making appropriate changes in sample preparation and applying dilution coefficients. In these cases, it is necessary to compare and verify the concentrations calculated from different dilutions.

Finally, the calculated range of the method (0.0000039%-0.01% w/v) was wide, which is acceptable for this method and meets the requirements. In this study, the LOQ was determined by analyzing samples with known concentrations of analyte and by establishing the minimum level at which the analyte can be determined with acceptable accuracy and precision (9). Therefore, the LOQ in this method was found to be 0.0000039% w/v of formaldehyde.

3.3. Recovery Estimation and Specificity

Accuracy was calculated as the recovery percentage by the assay of the known added amount of analyte in

the sample. Moreover, the recovery percentage was estimated as the ratio of the obtained results (calculated formaldehyde concentration) to the actual ones (spiked formaldehyde concentration).

The results of the three levels of concentration spiked to tetanus toxoid and DT vaccine, as well as the obtained recoveries are shown in tables 3 and 4.

Typically, the requirements for recovery in analytical methods for quantitative assays of impurities are between 90% and 110% (9, 13). Tables 3 and 4 show satisfactory recoveries obtained for 0.001% and 0.002% w/v concentrations for tetanus toxoid and DT vaccine, respectively. Recovery decreases from high to low concentrations, which is normal. The recovery for 0.0005% w/v concentration is a little more than 110%, which is not surprising because recovery deviates from the desired value at very small values (13). On the other hand, the free formaldehyde determination method in biological products is actually a limit test, and its specification is less than 0.02% w/v. Therefore, a slight deviation is acceptable and normal for recovery at very low values (such as 0.0005% w/v).

Overall, it can be concluded that the test method used in determining formaldehyde in the mentioned concentration has good accuracy and meets the acceptable percentage recovery requirements. In addition, the appropriateness of accuracy and precision in this method indicates the appropriateness of specificity.

3.4. Precision

Precision was calculated as the RSD between the calculated concentrations by the assay of the known added amount of analyte in the sample for three concentration levels.

The intra-precision or repeatability of the three levels of concentration for tetanus toxoid and DT vaccine are shown in table 3. Table 4 presents inter-day or inter-precision results of 15 replicates of determination on three different days (five replicates each day). As shown in table 3, the RSD values are less than 5% in high and medium concentrations and less than 10% in low concentrations. These RSD values are satisfactory and meet the requirements. Precision depends on concentration and, as mentioned, was measured at different concentrations within the working range. Usually, at lower concentrations, the precision decreases, and the RSD increases. At higher concentrations, better precision would be expected. The acceptance criteria may be widened when matrix effects are significant (13). In this regard, the RSD related to the accepted value for inter-precision was defined as less than 10%. As shown in table 4, all values for RSD in different concentrations and on different days are less than this value (10%). As a result, the free formaldehyde determination method has an acceptance precision in biological products and is repeatable.

Table 3. Accuracy and precision of the free formaldehyde determination method for tetanus toxoid and DT vaccine

Spiked Formaldehyde Concentration (%w/v)	Recovery for tetanus toxoid (%)	(RSD) Intra-precision for tetanus toxoid (n =5)	Recovery for DT vaccine (%)	(RSD) Intra-precision for DT vaccine (n =5)
0.001	102.03	3.74	104.01	2.03
0.002	107.78	2.24	109.39	4.79
0.0005	113.54	8.32	115.90	9.51

Table 4. Inter-day precision of the free formaldehyde determination method for tetanus toxoid and DT vaccine

Formaldehyde added (%w/v)	Recovery for tetanus toxoid (%) (n =15)	Inter-day precision (RSD) for tetanus toxoid (n =15)	Recovery for DT vaccine (%)	Inter-day precision (RSD) for DT vaccine (n =15) (n =15)
0.001	101.74	2.94	105.25	2.85
0.002	109.33	3.51	101.46	2.41
0.0005	108.54	9.94	113.33	5.99

4. Discussion

This study validated a compendial spectrophotometry method for determining free formaldehyde in biological samples of DT vaccine and tetanus toxoid antigen. According to the ICH and USP guidelines, different performance characteristics were investigated and calculated, and the results of each parameter were compared against the defined acceptance criteria.

The parameters used to validate this method were accuracy, intra-precision, inter-precision, linearity, range, and LOQ. Satisfactory precision (RSD%) of 2.24 and 4.79 for intra-day and 3.61 and 2.41 for inter-day were obtained in the medium concentration of linear range for tetanus toxoid and DT vaccine, respectively. The method's accuracy was also tested by spiking the analytes at three different concentration levels into a real sample. In the medium concentration of linear range, accuracy was 107.78% and 109.39% for tetanus toxoid and DT vaccine, respectively. The calibration curves showed great linearity for the analyte with a coefficient determination of 0.9999. Moreover, the high slope (266.7) in the regression equation obtained from the calibration curve confirmed the high sensitivity of this method. The free formaldehyde determination method was found to be linear in a wide range, and the LOQ value was found to be 0.0000039% w/v of formaldehyde.

The results show that the compendial method, with some consideration, can be a suitable and appropriate method for determining free formaldehyde in the DT vaccine and tetanus toxoid antigen. The developed method could achieve good linearity, suitable range, low LOQ, as well as acceptable accuracy and reproducibility. Furthermore, compared to other measurement methods (4, 15), this method is more practical and economical than chromatographic methods because of using spectrophotometry devices and proper analytical performance characteristics.

Authors' Contribution

Study concept and design: S. Z. and A .E.
Acquisition of data: S. Z.
Analysis and interpretation of data: S. Z. and A .E.
Drafting of the manuscript: S. Z.
Critical revision of the manuscript for important intellectual content: S. Z.
Statistical analysis: S. Z.
Administrative, technical, and material support: H. R. R. and A .E.
Study supervision: S. Z. and A .E.

Conflict of Interest

The authors declare that they have no conflict of interest.

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References

1. Jahrling PB, Stephenson EH. Protective efficacies of live attenuated and formaldehyde-inactivated Venezuelan equine encephalitis virus vaccines against aerosol challenge in hamsters. J Clin Microbiol. 1984;19(3):429-31.
2. Ogasawara N, Misaki M, Adachi S. Method for the determination of residual formaldehyde in an inactivated Zika vaccine formulated with aluminum hydroxide gel. Biologicals. 2019;58:73-5.
3. Standardization WECoB. Requirements for diphtheria, tetanus, pertussis and combined vaccines. WHO Tech Rep Ser. 1990;800:87-179.
4. Zali S, Jalali F, Es-haghi A, Shamsipur M. Determination of free formaldehyde in vaccines and biological samples using solid-phase microextraction coupled to GC-MS. J Sep Sci. 2013;36(24):3883-8.
5. Pharmacopoeia B. Free formaldehyde, method A. London: VA524; 2022.
6. Zurek G, Karst U. Liquid chromatography-mass spectrometry method for the determination of aldehydes derivatized by the Hantzsch reaction. J Chromatogr A. 1999;864(2):191-7.
7. Standards Toco. United States Pharmacopeia/National Formulary. General Chapter

<1226> Verification Of Compendial Procedures United State2020 [

8. WHO. WHO Expert Committee on Specifications for Pharmaceutical Preparations: World Health Organization; 2011.
9. Standards Toco. Validation Of Compendial Procedures United State: United States Pharmacopeia/National Formulary; 2020.
10. Administration FaD. Analytical Procedures and Methods Validation for Drugs and Biologics U.S. Department of Health and Human Services; 2015.
11. Agency EM. ICH guideline Q2(R2) on validation of analytical Procedures. Guideline Q2A: Text on Validation of Analytical Procedures: International Conference on Harmonization; 2022.
12. WHO. WHO Expert Committee on Specifications for Pharmaceutical Preparations: Fiftieth Report: World Health Organization; 2016.
13. Drugs UNOo, Laboratory C, Section S. Guidance for the Validation of Analytical Methodology and Calibration of Equipment Used for Testing of Illicit Drugs in Seized Materials and Biological Specimens: A Commitment to Quality and Continuous Improvement: United Nations Publications; 2009.
14. Ingle Jr JD, Crouch SR. Spectrochemical analysis. 1988.
15. Bagheri H, Ghambarian M, Salemi A, Es-Haghi A. Trace determination of free formaldehyde in DTP and DT vaccines and diphtheria-tetanus antigen by single drop microextraction and gas chromatography-mass spectrometry. J Pharm Biomed Anal. 2009;50(3):287-92.