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Short communication

Determining trace amounts and the origin of formaldehyde impurity in *Neisseria meningitidis* A/C/Y/W-135-DT conjugate vaccine formulated in isotonic aqueous 1× PBS by improved C18-UPLC method

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ABSTRACT

The ability to accurately measure and report trace amounts of residual formaldehyde impurity in a vaccine product is not only critical in the product release but also a regulatory requirement. In many bacterial or viral vaccine manufacturing procedures, formaldehyde is used either at a live culture inactivation step or at a protein de-toxification step or at both. Reported here is a validated and improved C18-UPLC method (developed based on previously published C-8 HPLC method) to determine the traces of formaldehyde process impurity in a liquid form *Neisseria meningitidis* A/C/Y/W-135-DT conjugate vaccine formulated in isotonic aqueous 1× PBS. UPLC C-18 column and the conditions described distinctly resolved the 2,4-DNPH–HCHO adduct from the un-reacted 2,4-DNPH as detected by TUV detector at 360 nm. This method was shown to be compatible with PBS formulation and extremely sensitive (with a quantitation limit of 0.05 ppm) and aided to determine formaldehyde contamination sources by evaluating the in-process materials as a track-down analysis. Final nanogram levels of formaldehyde in the formulated single dose vialed vaccine mainly originated from the diphtheria toxoid carrier protein used in the production of the conjugate vaccine, whereas relative contribution from polysaccharide API was minimal.

1. Introduction

Formaldehyde acts as an electrophile and can react with negatively charged macromolecules such as DNA, RNA and polysaccharides to form reversible adducts or irreversible cross-links. Therefore, it is considered a carcinogenic, mutagenic and genotoxic substance [1]. Ramon [2], Glenny and Hopkins [3] showed that diphtheria toxin can be detoxified by formalin treatment. Since then, formaldehyde (HCHO) has been used in many viral and bacterial inactivation and/or vaccine manufacturing or disinfection procedures [4,5]. According to the information by Children's Hospital of Philadelphia [6] and other data available on http://www.cdc.gov/vaccines/vac-gen/additives.html (browsed 2nd January 2015) (Ingredients of vaccines-Fact sheet CDC) it is known that many vaccines that are currently licensed in Unites States contain different levels of residual formaldehyde impurity. Regulatory agencies often specifically advise and request the procedural measures from manufacturers to exactly determine the residual levels of formaldehyde in the final vaccine product. For example in a formalin-killed virus based animal vaccine, USDA limit on formaldehyde content is 0.74 g/l as determined by ferric chloride test [7]. Although there is no clear guidance limit on HCHO in human vaccines, USFDA has recently published a risk assessment on residual formaldehyde in infant vaccines by a remarkable pharmacokinetic modeling approach [8] indicating exogenously applied formaldehyde content (anywhere between 0.4 and 100 μg per 0.5 ml dose) of current infant vaccines is safe. Although there are a number of methods available to measure such formaldehyde traces, it is vital to choose a method that does not interfere with the formulation chemistry and the composition of a particular final product. Moreover since formaldehyde is not easily ionizable, sensitive techniques such as gas chromatography or mass spectrometry could not be applied to micro-analyze this molecule.

Neisseria meningitidis is a Gram-negative encapsulated bacterium that causes meningococcal meningitis and septicaemia, and is classified into 13 serogroups based on expression of cell

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surface antigenic capsular polysaccharide chemical structures and by serological properties. Serogroups A, B, C, W-135, X and Y cause the majority of disease globally. Polysaccharide and polysaccharide–protein conjugate licensed vaccines are available for serogroups A, C, W-135 and Y [9]. In pneumococcal, meningococcal and *Haemophilus influenzae* type B polysaccharide–protein conjugate vaccine manufacture [10], where carrier proteins such as tetanus toxoid (TT) or diphtheria toxoid (DT) are used, there may be steps where formaldehyde is used not only in detoxification of carrier proteins, but also in respective bacterial inactivation stage during up-stream polysaccharide preparation. Down-stream purification steps of conjugate vaccine preparation, remove formaldehyde impurity down to nanogram or microgram quantities per dose of final product. Even though impurities are at such a low level, the relative origin of formaldehyde impurity in the final product, whether from polysaccharide or from carrier protein or from both still needs clarification. This information can be pivotal to vaccine manufacturers for process improvement in order to minimize the formaldehyde impurity levels in the final product.

A reverse phase HPLC method for the determination of low level formaldehyde in a solid drug substance was reported by Soman et al. [11], where formaldehyde reacted with 2,4-dintirophenylhydrazine (DNPH) to form a HCHO–DNPH derivative that was measured at 360 nm on a diode-array detection. Based on their method a modified and improved UPLC microanalysis method was developed that determines nanogram quantity of formaldehyde in liquid vaccine formulated in 1× PBS at 0.5 ml dose. In sample preparation the 2,4-DNPH derivatization reaction volume is adjusted to 1 ml to match final product dose volume in this method. Also UPLC C18 reverse phase column was used to improve the resolution and to minimize the run time to 10 min. 1× PBS background and spiking with known formaldehyde in product sample matrix were studied to understand the suitability of the method for this vaccine's formulation chemistry. Microanalysis of formaldehyde levels by this method in polysaccharide, DT, mono-conjugates, and final vialed vaccine led to the observation that the majority of residual formaldehyde in the final vialed product (A/C/Y/W-135-DT conjugate vaccine) is originating from DT.

2. Materials and methods

2.1. Safety concerns

Formaldehyde is considered toxic. All samples and reactions containing formaldehyde were handled under fume hood. During UPLC runs column-flow through was collected separately in hazardous waste containers and disposed using safety procedures.

2.1.1. Materials

2,4-Dinitrophenyl hydrazine (cat# D199303), phosphoric acid solution (85% solution, cat# 345245) were purchased from Sigma (St. Louis, MO, USA). 37% Formaldehyde solution in water (A.C.S. reagent cat# F79-500), Acetonitrile and HPLC grade water were purchased from Fisher Scientific (Pittsburgh, PA, USA). Formaldehyde standards, phosphoric acid and DNPH reagent solutions were prepared as mentioned in Soman et al. [11].

2.1.2. Vaccine preparation

Information about *Neisseria meningitidis* A/C/Y/W-135-DT conjugate vaccine preparation and formulation, details on polysaccharide and protein quantification were published elsewhere [12]. During polysaccharide production, individual serogroup fermentation culture batches were either heat inactivated (56 °C, 45 min) or 0.5% v/v formalin inactivated before harvesting. DT (Serum Institute of India) was detoxified using 0.6% (v/v) formalin at 36 ± 1 °C for 6 weeks. DT was further polished by Octyl sepharose hydrophobic interaction column chromatography at JN facility.

2.2. Chromatographic-conditions

Waters UPLC Acquity H-class system with TUV detector that runs on Empower-2 software was used. Waters UPLC BEH C18, (2.1 mm ID × 50 mm length) column with Waters VanGuard (2.1 mm ID × 5 mm length) guard column was used with mobile phase – water:acetonitrile (55:45 v/v) at a flow rate of 0.2 ml/min. Dual-wavelengths were set one at 360 nm and the other at 280 nm. Total run time of 10 min was optimized. The mobile phase (55% water and 45% acetonitrile) was the same as in the method of Soman et al. Using low concentration (0.1 μg/ml) of formaldehyde in the sample, the flow rate was standardized to 0.2 ml/min on the UPLC C-18 column conditions, to have comfortable resolution between DNPH (R.T. ~ 2 min) and DNPH–HCHO (R.T. ~ 4.5 min) peaks.

2.3. Sample details

Two vials of final product each from two lots (JN-NM-001 and 4G13F01) were tested. From a single vial, 500 μl vaccine was pulled out using 1-ml syringe with a needle. Apart from final vialed material, as part of track-down analysis, in-process materials such as purified polysaccharide, source DT, polished DT and individual mono-conjugates were tested for HCHO levels at a suitable normalized concentration of either polysaccharide or protein in the sample. Polysaccharides of serogroups A, C, W and Y that were manufactured from formaldehyde inactivated (FI) culture batches 10Sep12A, 15Oct12C, 30Sep12W, and 08Oct12Y respectively were tested at 1 mg/ml PS. Source DT and polished DT were tested at a sample concentration of 16 and 32 μg/ml protein as estimated by BCA assay. Samples from two lots of polished DT (Lot-1DTP and Lot-2 DTP) that were used for making the two lots of vaccines JN-NM-001 and 4G13F01 respectively were tested to compare the Octyl sepharose polishing efficiency to remove HCHO and to verify the column purification method differences for the two lots.

2.4. Sample preparation and reaction mixture

To a 500 μl of sample (mentioned above), transferred to 1.5 ml micro-centrifuge tube, 10 μl of 5 N phosphoric acid solution and 100 μl of 1 mg/ml DNPH solution were added and mixed immediately by vortexing. The blank contained 500 μl water or 1× PBS, 10 μl 5 N Phosphoric acid, and 100 μl 1 mg/ml DNPH. A timer was set for 30 min and each sample vortexed briefly after every 5 min point during the 30 min reaction. 390 μl of water was added to each sample (including the blank) immediately after the 30 min reaction. Samples were again vortexed briefly to mix and 500 μl from each was transferred to a HPLC vial. Duplicate (15 μl) injections were made for each sample.

2.5. Method validation

In order to validate the proposed UPLC method, validation characteristics such as accuracy, specificity, detection limit, quantitation limit, linearity and solution stability were considered as per ICH guidance for industry Q2B and Q2 (R1) [13,14]. A linear relationship between HCHO and 2,4-DNPH was established in two sets of ranges to arrive at formaldehyde standard curves of one low range using (i) 0.03, 0.04, 0.05, and 0.1 μg/ml, the other high range using (ii) 0.25, 0.5, 1.0, 2.0 μg/ml of HCHO in the derivatization reaction to a final volume of 1 ml. HCHO concentration (μg/ml) vs average peak area (μV s) of duplicate injections were plotted. Solution stability on these three standard concentrations were tested for <2 h, 4 h, 12 h and 24 h time points at 22 ± 2 °C laboratory temperature.

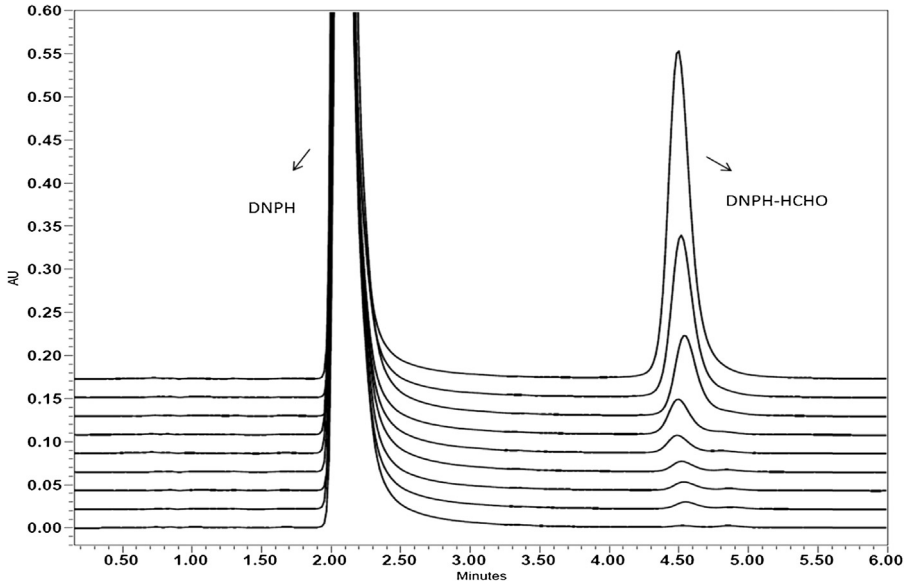

Fig. 1. UPLC C-18 overlay of standard formaldehyde profiles with increasing concentrations (Table 2), where bottom most profile being water blank. Formed DNP–HCHO adduct exhibited increased linearity of peak (retention time approximately 4.5 min) area at 360 nm.

Three spiking concentrations (0.25, 0.5 and 1.0 ppm) in the product sample matrix were tested for the interference and recovery in triplicate injections on each concentration.

2.6. Using Empower-2 process method, peak areas were obtained. From the areas derived (if any), from these samples, used the standard curve formula to determine concentration of formaldehyde in the sample.

3. Results and discussion

A clear linearity (Fig. 1) was observed in the 360 nm absorption at ~4.5 min, with increasing concentrations of 0.03, 0.04, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 μg/ml (ppm) HCHO standard used in the sample preparation. Samples of 1× PBS were used to verify any interference or noise level. A control 1 μg HCHO spiked sample was used to confirm the DNPH–HCHO product (Fig. 2) peak absorption at 360 nm. The standard curve points were utilized to plot two calibration curves (low range and high range) to facilitate using them for low levels or high levels expected in the test samples (Table 1). When product matrix obtained from vial was used for testing three spiking concentrations of 0.25, 0.5 and 1 ppm generated profiles showed linearity and precision (Fig. 3). Quantitative % recovery (calculated based on the peak areas of triplicate injections against three spiked concentrations) respectively was 104 ± 1.9, 98 ± 1.0

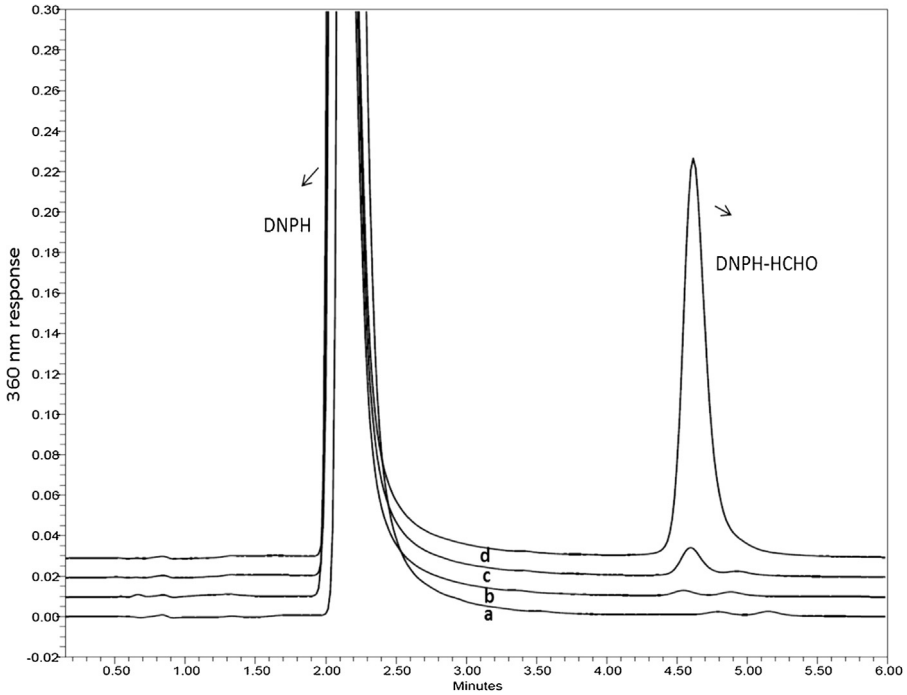

Fig. 2. UPLC C-18 profile comparison overlay with samples of (a) water blank; (b) 1× PBS blank; (c) 500 μl vial component; (d) 500 μl vial component +10 μl of 10 μg/ml HCHO spiked.

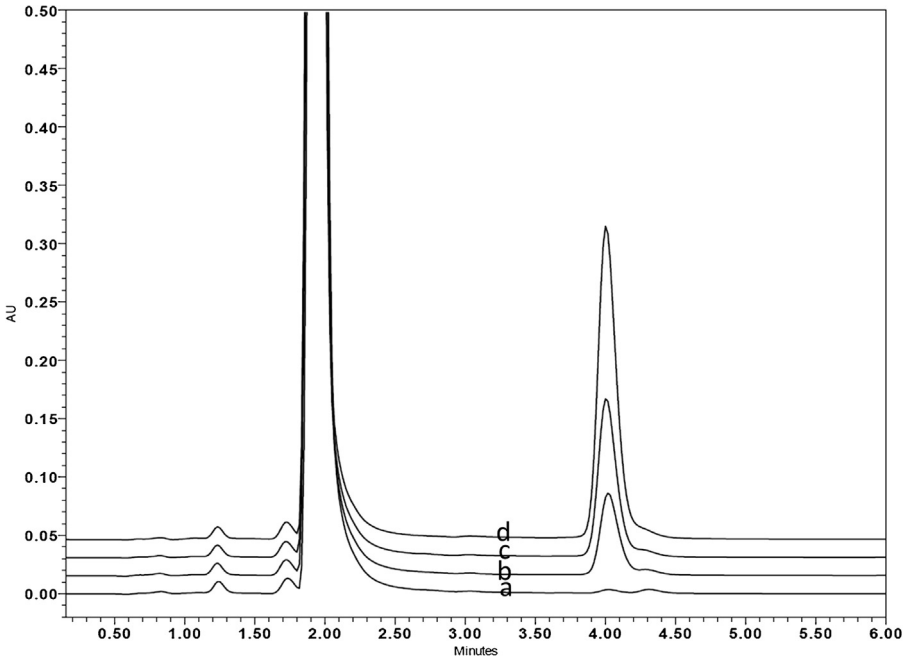

Fig. 3. Overlay UPLC C-18 profile comparison with formaldehyde spike in product sample matrix (a) blank; (b, c, d) 0.25, 0.5, 1 ppm HCHO spiked respectively. Quantitative % recovery was calculated based on peak areas of triplicate injections of each spike.

Table 1
Increasing concentrations of formaldehyde were used to generate low and high range standard curves by 2,4-DNP C-18 reverse phase UPLC assay. Average peak areas of duplicate injections were used to plot.

Sample	Concentration (μg/ml) ppm	Mean area (μV s)
1	0	0
2	0.03	90,444.5
3	0.04	112,706
4	0.05	136,422.5
5	0.1	239,141.5
6	0.25	512,844.5
7	0.5	1,153,819.5
8	1.0	2,335,322.5
9	2.0	4,679,784.0
Trendline formula for low range samples 1–5	$y = 2E+06x + 1875.8$	$R^2 = 0.9997$
Trendline formula for high range samples 6–9	$y = 2.3294x - 0.0237$	$R^2 = 0.9998$

and 98 ± 1.1 (% RSD of 0.19, 0.05 and 0.06 respectively) indicated good precision. Solution stability runs indicated that the HCHO–2,4 DNP adduct is stable up to 24 h (data not presented) at observed temperature of 22 ± 2 °C.

Purified four serogroup PSs (at 1 mg/ml) were tested for HCHO content and the results were tabulated in Table 2. Although these polysaccharides were prepared from fermentation cultures that were formaldehyde inactivated during upstream process step they showed very low levels of formaldehyde impurity at a final purified stage.

Table 2
A/C/W/Y Four serogroup polysaccharides at 1 mg/ml PS concentration were tested for their formaldehyde content and Mean ± S.D. of duplicate injections were presented below.

Sample	Mean area under curve (V s)	Formaldehyde (μg/ml) mean ± S.D.
10Sep12A (FI)	0.035	0.0097 ± 0.001
15Oct12C (FI)	0.033	0.0087 ± 0.001
30Sep12W (FI)	0.026	0.0058 ± 0.001
08Oct12Y (FI)	0.026	0.0059 ± 0.001

FI: formalin inactivated culture batches.

Multiple samples of source DT or polished DT (batch number 12Dec12-DTP) were tested at normalized concentration of protein at 16 and 32 μg/ml and the mean ± S.D. values of duplicate injection results were tabulated in Table 3. Overall supplied DT contained 11.5 ± S.D. μg/mg protein and polished DT samples exhibited 2.0 ± S.D. μg/mg protein of HCHO indicating polishing step efficiently removed majority (70–80%) of HCHO impurity. Two lots (Lot-1 and Lot-2 DTP) of polished DT tested showed approximately 5 fold improvement in terms of HCHO removal in Lot-2. (The details of Octyl sepharose polishing method differences are not within the scope of this report and hence not discussed in this report.)

Individual serogroup PS-DT mono-conjugates: A-DT, C-DT, W-DT and Y-DT samples were tested for their formaldehyde impurity levels at normalized PS concentration of 0.2 mg/ml and mean ± S.D. values obtained from duplicate injections were tabulated in Table 4. Their HCHO levels were closely ranging from 0.5 to 0.6 μg/mg PS.

Two lots of vialed vaccine were tested for the formaldehyde levels in them. Results were tabulated as Table 5. A two to three fold difference in levels of HCHO is observed between the two lots. The Old Lot JN-NM-001 used Lot-1 DTP (Table 3) when Octyl sepharose column purification method was in development stage and hence the level of HCHO was higher in this lot of DTP.

Detection Limit (DL) and Quantification Limit (QL) were determined according to ICH Q2 (R1) [14]. DL (0.015 μg/ml) and QL (0.046 μg/ml) were expressed respectively by the following formulae $DL = 3.3\sigma/S$ and $QL = 10\sigma/S$, where σ: residual standard deviation of the response and S: slope of the standard curve. The DL and QL values were calculated from the standard curve containing the four lowest formaldehyde amounts. The relative standard deviation of the response was calculated using the STEYX function in EXCEL as 0.0109, using the four lowest concentrations of formaldehyde. Slope (m) was derived from the calibration curve equation $y = mx + b$. Calibration curves, equations and R^2 values generated using Microsoft EXCEL are shown in Table 1.

Table 3
Duplicate samples of DT before (source DT) and after column purification (DT polished) were tested for formaldehyde levels in them at different concentrations of protein in the sample. Each sample had duplicate injections. Formaldehyde content per mg of protein (mean ± S.D.) on duplicate injections were presented below.

Sample (protein concentration)	Area under curve (V s)	Formaldehyde (μg/ml)	HCHO mean ± S.D. (μg/mg protein)
DT (16 μg/ml)	0.44	0.20	12.48 ± 0.14
DT (32 μg/ml)	0.87	0.39	12.03 ± 0.02
12Dec12DTP (16 μg/ml)	0.09	0.03	2.12 ± 0.005
12Dec12DTP (32 μg/ml)	0.18	0.07	2.18 ± 0.04
Lot-1DTP (1 mg/ml)	11.81	4.94	4.94 ± 0.26
Lot-2DTP (1 mg/ml)	2.31	0.97	0.98 ± 0.2

Table 4
Duplicate samples of four serogroup monoconjugates at normalized 0.2 mg/ml protein concentration were tested for their formaldehyde content and mean values of duplicate injections presented.

Sample	Area under curve (V s)	Mean formaldehyde (μg/ml)
A-DTmonoconjugate (FI) (0.2 mg/ml PS, 0.328 mg/ml protein)	1.15	0.49
C-DT monoconjugate (FI) (0.2 mg/ml PS, 0.336 mg/ml protein)	1.30	0.55
W-DT monoconjugate (FI) (0.2 mg/ml PS, 0.216 mg/ml protein)	1.01	0.43
Y-DT monoconjugate (FI) (0.2 mg/ml PS, 0.350 mg/ml protein)	1.55	0.65

Table 5
Two vials from each of two lots of vialed vaccine and two samples of 1× PBS were tested for their levels of formaldehyde. HCHO content (mean ± S.D.) from duplicate injections were tabulated below.

Sample	Area under curve (V s)	Mean ± S.D. HCHO (μg/ml)
1× PBS	0.027	0.0064 ± 9.84E-06
JN-NM-001 vial (HI)	0.413	0.1825 ± 0.02
Vial 4G13F01 (FI)	0.154	0.0605 ± 0.0013

In a generalized scheme of polysaccharide-protein conjugate vaccine manufacturing, active pharmaceutical ingredients (APIs) such as polysaccharides and carrier proteins are produced in separate fermentation batches. Both the fermentation cultures most commonly are inactivated by formalin before the upstream harvesting step. Carrier toxin proteins are generally de-toxified by prolonged (4–5 weeks in 0.1% formalin) formalin treatment. Downstream purification steps (such as polishing by Octyl sepharose column and/or diafiltration) on both these partner APIs remove the majority of contaminating formaldehyde. Later these partner APIs are generally activated, individually conjugated followed by multiple diafiltration steps and finally formulated as a conjugate vaccine product. Improved UPLC C18 method, helped not only in determining the traces of HCHO in the final product, but also helped to visualize the details of relative contamination source(s) after analyzing in-process samples at various stages. Method improvements included upgrading HPLC to UPLC resolution, reduced run-time and liquid drug product compatible sample preparation. This method was found to be extremely sensitive (with a quantitation limit of 0.05 ppm), reproducible and compatible with 1× PBS formulation chemistry. Although the track-down analysis showed that the majority of formaldehyde impurity is originated from the DT that is used as carrier protein, it was not determined why this small molecular contaminant (HCHO) is still carried to the final product despite polishing and many diafiltration steps. The electrophilic nature of HCHO and anionic nature of DT and the polysaccharide, resulting irreversible adduct formations may be possible reasons.

4. Conclusions

Validated UPLC C-18 2,4 DNP method described in this report, is an upgraded and modified version of a previously published HPLC C-8 method, and works for the microanalysis of formaldehyde in a 1× PBS formulated aqueous liquid vaccine without interference. This method is highly sensitive, reproducible and enabled to identify the relative formaldehyde contamination source in the vaccine manufacturing process.

Conflict of interest

The authors have no competing financial conflicts of interests in publishing this material.

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