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UPLC Determination of Process Impurity Hydrazine in *Neisseria meningitidis* A/C/Y/W-135-DT Conjugate Vaccine Formulated in Isotonic Aqueous 1x PBS

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Abstract

Purpose: To develop and validate a rapid and sensitive UPLC method as a limit test to determine traces of hydrazine process impurity in a *Neisseria meningitidis* A/C/Y/W-135-DT conjugate vaccine formulated in isotonic aqueous 1x PBS.

Methods: Hydrazine was derivatized in a single step with salicylaldehyde at 60°C for 60 minutes and the adduct was resolved from the un-reacted salicylaldehyde on a Waters UPLC BEH C18, (2.1 mm ID x 50 mm length) column with mobile phase- water: acetonitrile (40:60 v/v) at a flow rate of 0.2 mL/minute and detected by TUV detector at 209 nm. Sample matrix was prepared from final PBS formulated vaccine to study the % spike recovery.

Results: UPLC C-18 column and optimized conditions achieved better sensitivity and resolution than previously reported HPLC method. Clear linearity (r^2>0.99) was exhibited with hydrazine standard (in PBS) at concentrations ranging from 0.25 to 12.5 ppm. Spike recovery of the method was >99%. Detection limit (DL) and quantitation limit (QL) were 0.08 ppm 0.25 ppm respectively. Hydrazine impurity in the formulated single dose vialed final product is lower than detection limit by this method.

Conclusions: Results indicated that this method is sensitive, rapid, reproducible, accurate and compatible with PBS formulation.

Introduction

Meningococcal meningitis is caused mainly by *Neisseria meningitidis* serogroups A, B, C, W-135, X and Y. Licensed Polysaccharide and polysaccharide-protein conjugate vaccines are available for serogroups A, C, W-135 and Y [1,2]. In the conjugation process, the carrier proteins can be activated by an EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) mediated hydrazine reaction (Figure 1) to generate active hydrazide moieties on the protein molecule [3, 4]. Since hydrazine is toxic to humans, acceptance criteria for levels of hydrazine in pharmaceutical substances and analytical methods for its determination have been recently reviewed [5]. Hence sensitive methods to determine hydrazine in final vaccine products, compatible with formulation chemistry are important in quality control, quality assurance and release of the product.

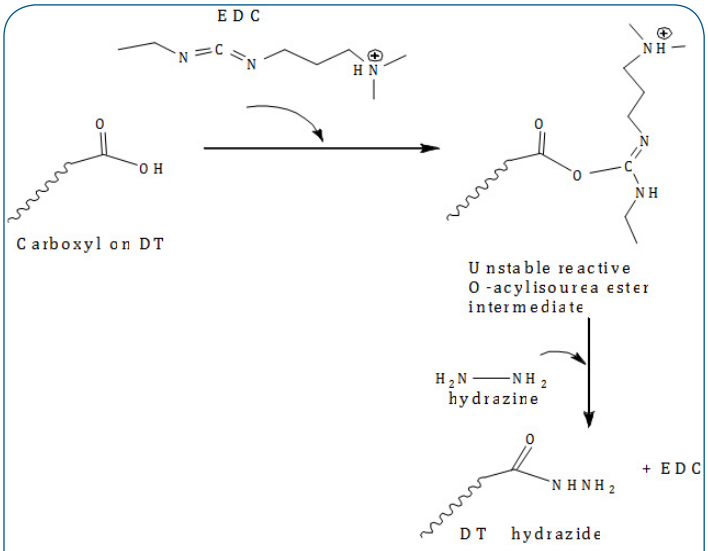

Figure 1: Scheme of EDC mediated hydrazine activation of diphtheria toxoid (DT) protein. Glutamic acid or aspartic acid residues on the protein react in the presence of water soluble organic catalyst EDC to facilitate the hydrazine reaction, to form hydrazide active group on the protein. Unreacted excess hydrazine and EDC are removed by diafiltration after the activation reaction

George et.al [6] published a HPLC method with limit of detection 10 ppm to determine trace hydrazine levels in a drug substance. Based on their method, this report is an improved UPLC method that has been developed to be compatible with a liquid meningococcal conjugate vaccine formulated in isotonic 1xPBS pH 7.4. Bastos et.al [7] recently published a more sensitive differential pulse polarographic method to determine hydrazine in meningococcal C conjugate vaccine intermediary product. However, liquid chromatography tools are more commonly used by the polysaccharide-protein conjugate vaccine industry to address various analytical needs such as molecular size estimations of polysaccharide, protein and the conjugates. UPLC gives not only dramatic improvement in speed, resolution and

sensitivity of analysis but also decreases solvent consumption [8]. The reported UPLC method takes advantage of existing equipment and provides better resolution and quantitation than the previously described HPLC method. **Novelty of the work**: A rapid and more sensitive UPLC method is developed and validated as limit test to determine traces of hydrazine process impurity in a ***Neisseria meningitidis* A/C/Y/W-135-DT** conjugated vaccine formulated in PBS. The UPLC method achieved greater resolution and sensitivity with detection limit of 0.08 ppm compared to previously reported HPLC method that detected up to 10 ppm. Run time for this UPLC method is 5 minutes whereas previous HPLC method was 20 minute long.

Materials and Methods

Anhydrous Hydrazine $_{\text{(catalogue \#211155)}}$ was purchased from Sigma (St. Louis, MO, USA). Salicylaldehyde (catalogue# $_{\text{AC13260-2500}}$) was purchased from Acros Organics, New Jersey, USA. Acetonitrile and HPLC grade water were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Vaccine: Hydrazine activated diphtheria toxoid (DT) was used in the preparation of the tetravalent A/C/Y/W-135-DT vaccine used in this work. Further Information about *Neisseria meningitidis* A/C/Y/W-135-DT conjugate vaccine preparation and formulation, were published elsewhere [9].

Hydrazine stock solution: 10 mL of 5M hydrazine pH 6.5 stock is prepared from 1.6 mL of anhydrous hydrazine with density 1.021 g/ml by adjusting pH on ice-bath in a fume hood. 5M hydrazine stock is calculated to contain 163,360 ppm, which was diluted further to 1000 ppm hydrazine working solution in HPLC grade water.

Preparation of salicylaldehyde working solution: Salicylaldehyde (85µl) from supply bottle was added to a 10 mL volumetric flask. Made up the volume with HPLC grade water to the 10 mL mark, capped and mixed well. This gives 10,000 ppm salicylaldehyde working solution.

Sample matrix: 0.5 mL dose final vaccine product was passed through a 10kDa molecular weight cut-off spin filter by centrifuging on a table top centrifuge at 13,000 rpm for 10 minutes, and filtrate is considered as sample matrix.

Sample preparation: To a 50 µL sample (1 x PBS or a sample matrix blank), 20 µL of acetonitrile and 50 µL of salicylaldehyde working reagent were added, briefly vortexed to mix and kept at 60°C water bath for 1hour. After the incubation, tubes were taken out of the water bath, vortexed briefly to mix and 100 µL of the sample was pipette into a UPLC maximum recovery sample vial and placed in the auto-sampler compartment of the UPLC system. Duplicate or triplicate injections were applied for precision and spiking samples.

Chromatographic-conditions: A 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 x 50 mm length) column with Waters VanGuard (2.1 mm ID x 5 mm length) guard column was used with mobile phase- water: acetonitrile (40:60 v/v) at a flow rate of 0.2 mL/minute. Dual-wavelengths were set, one at 209 nm and the other at 280 nm. Total run time of 5 minutes was optimized.

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 and passed through a 10 kDa spin filter. Filtrate was tested for hydrazine content.

Method validation: Characteristics tested included accuracy, specificity, detection limit, quantitation limit, linearity and solution stability according to ICH guidance for industry Q2B and Q2 (R1) (10-11). A range of 0.25 to 12.5 ppm hydrazine (concentrations 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 7.5, 10.0 and 12.5 ppm) in 1xPBS were used for calibration curve. Hydrazine concentration (ppm or µg/mL) vs average peak area (µV*sec) of duplicate injections were plotted. Accuracy, solution stability, intermediate precision between two analysts, intra-day and inter-day precision, of the method was assessed using four spiking concentrations (1, 2, 5 and 10 ppm) in the product sample matrix and percent recovery on each spike was measured. Solution stability was measured for <2h, 4h, 12h and 24h time intervals leaving the sample in the autosampler compartment at 22±2°C laboratory temperature and repeating injections. Peak areas were compared with an intra-day (4h apart) and inter-day (24h apart) repeated assays of the four spike concentrations. Waters Empower-2 processing method was used to evaluate peak areas.

Results

After several runs of experiments hydrazine- salicylaldehyde derivatization at 60°C, 60 minutes incubation step has been optimized. To assess the performance of the method linearity, precision and accuracy were measured. Clear linearity (R^2 >99.9, N=9) (Figure 2) was observed with hydrazine-salicylaldehyde adduct (209 nm) absorption at ~3.75 min, with increasing concentrations of 0.25, 0.5, 1.0, 2.0, 3.0, 5.0, 7.5, 10.0 and 12.5 ppm hydrazine standard in 1x PBS pH 7.4.

To avoid large molecular vaccine components such as polysaccharide, protein and polysaccharide-protein conjugates to interfere with the method, a 10 K molecular weight cut-off spin filtration step was introduced in sample matrix preparation. Four different concentrations1, 2, 5 and 10 ppm (Figure 3) were used to spike the sample matrix obtained from final vaccine product to confirm the peak absorption and % recovery (Table 1). Quantitative % recovery (calculated based on the peak areas of triplicate injections against three spiked concentrations) was >99% in all the four spike concentrations. Between two analysts (data not presented) and Intra-day and inter-day precision (Table 2) indicated good precision and compatibility of the method with sample matrix. At the lowest spike concentration of 1 ppm higher % CV was observed than for 2, 5 or 10 ppm hydrazine concentrations. Solution stability runs indicated that the adduct is stable up to 24 h at observed temperature of 22±2°C (data not presented).

Discussion

Since hydrazine is toxic and a possible human carcinogen [12], its presence is limited in some of the drug substances, for example, US pharmacopoeia (USP) limit for hydrazine level in a vasodilating drug substance Hydralazine hydrochloride is 10 ppm (method of analysis is by HPLC-Benzaldehyde derivative) and for an antiseptic Povidone hydrazine limit (both USP and Eu.Ph) is 1ppm (method of analysis is TLC of Salicylaldehyde derivative) [5].

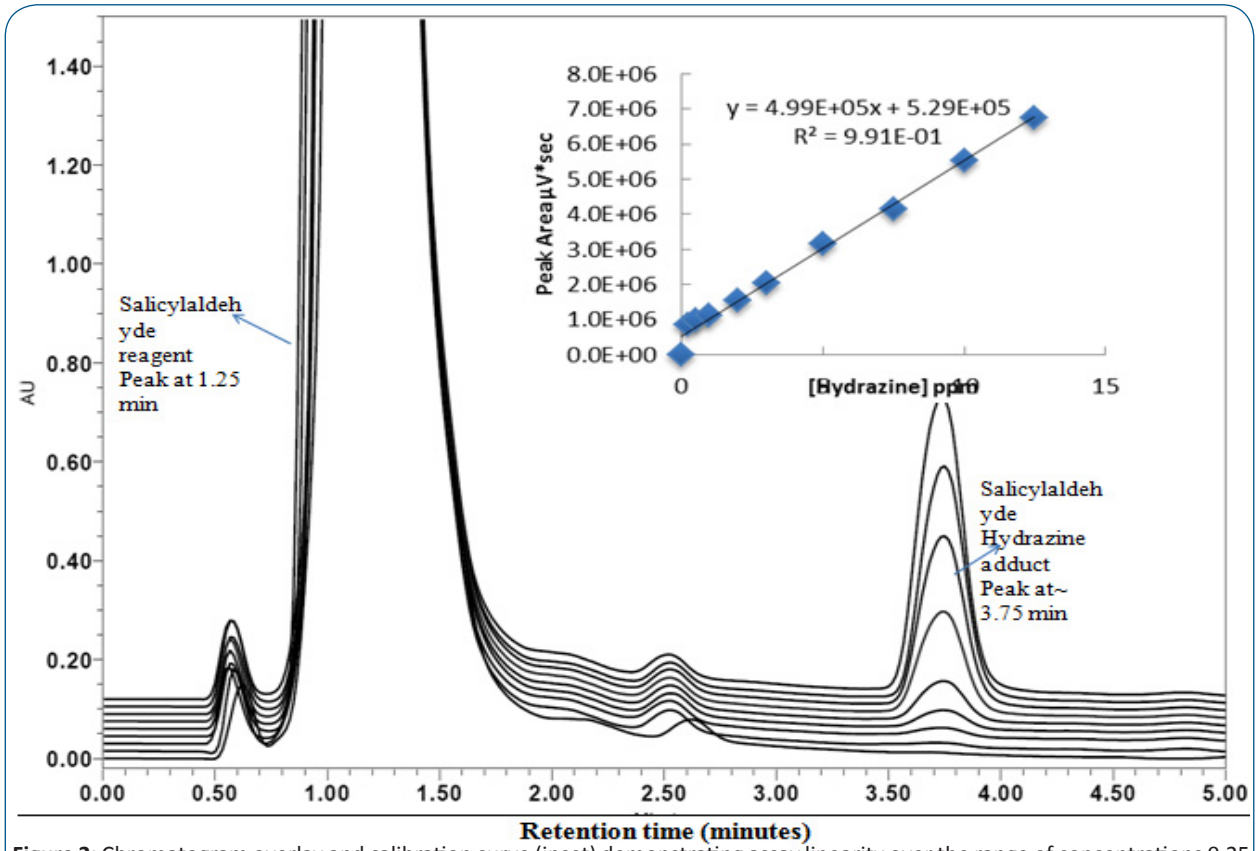

Figure 2: Chromatogram overlay and calibration curve (inset) demonstrating assay linearity over the range of concentrations 0.25 to 12.5 ppm. Bottom most profile on the chromatographic overlay represents PBS blank. Salicylaldehyde reagent peak was at 1.25 min and salicylaldehyde-hydrazine adduct peak at ~3.75min. Assay accuracy is poorer at lower concentration points

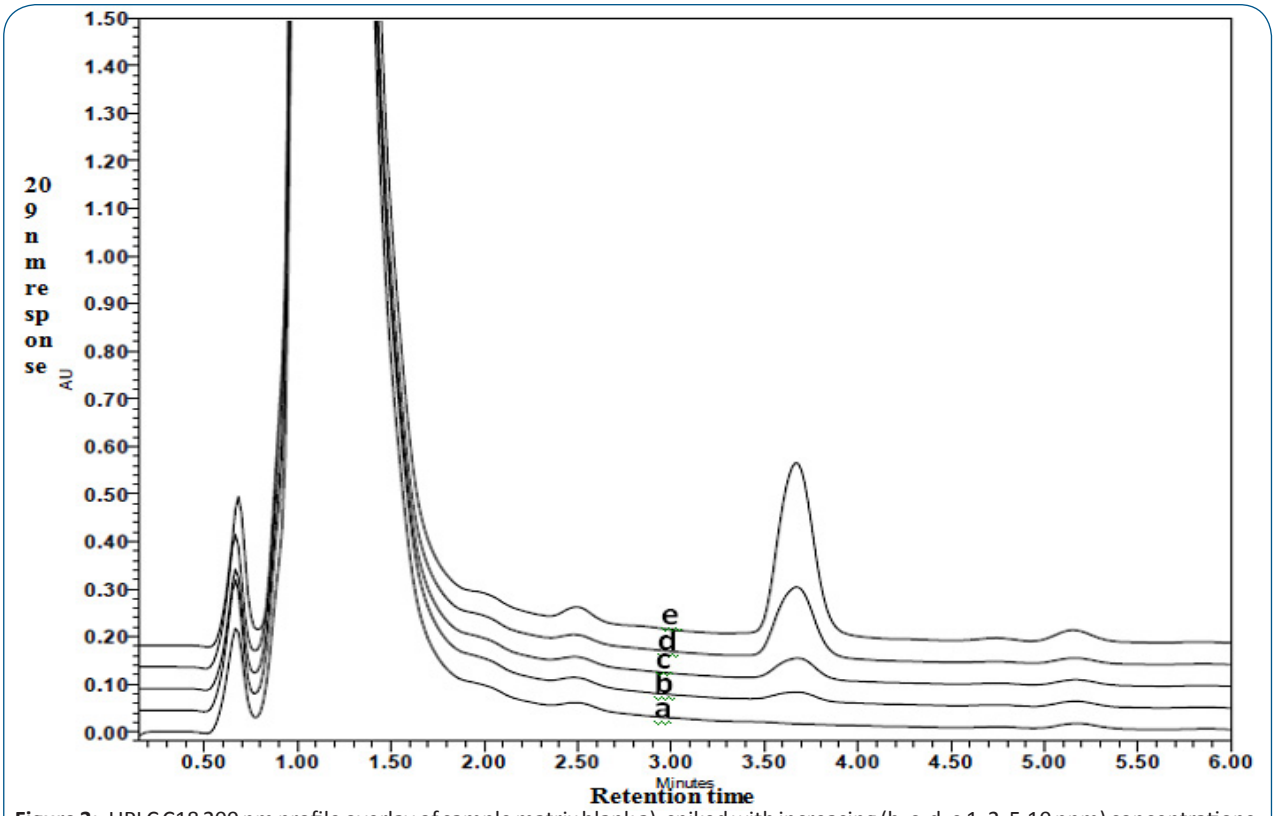

Figure 3: UPLC C18 209 nm profile overlay of sample matrix blank a), spiked with increasing (b, c, d, e 1, 2, 5,10 ppm) concentrations of hydrazine

Table1: Sample matrix was spiked with four different concentrations 1, 2, 5 and 10 ppm hydrazine and % recovery and % RSD were calculated on triplicate injections. Recovery was above 99% in all tested concentrations indicating sample matrix compatibility and the assay accuracy

Ppm Spiked	Mean Area±SD of triplicate injections (V*sec)	Ppm found	% recovery	% RSD
1	1255247±6614	1.10	110.0	0.53
2	1629180±12330	2.06	103.1	0.76
5	3046445±58583	4.95	99.05	1.92
10	6070837±11456	10.10	101.0	0.19

Table 2: UPLC Assay exhibited inter-day and intra-day intermediate precision. Tabulated are the mean±CV of the peak areas of respective hydrazine amounts assayed either 4h or 24h apart using proposed assay. 1 ppm showed higher % CV during observation than 2, 5 or 10 ppm hydrazine concentrations tested

Hydrazine (ppm) assayed	mean±% CV peak areas (triplicate injections)			
	Intra-day precision		Inter-day precision	
	4h apart		24h apart	Mean difference
1	1530791± 8.4	1399439±2.2	1458364±1.7	72427
2	1866946±0.9	1839042±0.7	1839102±0.7	27844
5	3019654±1.0	3229903±0.6	3660186±0.6	640532
10	4993753±0.9	5356213±1.5	5456113±1.3	462360

Detection Limit (DL) and Quantification Limit (QL)were determined according to ICH Q2 (R1). DL (0.08 ppm) and QL (0.25 ppm) were expressed respectively by the following formulae DL=3.3σ/S and QL=10σ/S, where **σ**: residual standard deviation of the response and **S**: slope of the standard curve. Slope (m) was derived from the calibration curve equation y= mx+b**.** Calibration curve, equation and R^2 values generated using Microsoft EXCEL

Down- stream purification steps (such as diafiltration) after protein activation, removed the majority of process impurities of un-reacted hydrazine, EDC, and other residual salts. However it was safer to verify the removal of this hazardous impurity in the final vaccine product as a release criterion. This improved UPLC C18 method, helped to verify sub ppm levels if any in the final product. Method improvements included upgrading HPLC to UPLC resolution, reduced run-time (to 5 minutes from 20 minutes) and optimizing liquid drug product compatible sample preparation (60°C, 60 min heating step). Shorter run time allows for more samples to analyze per day. This method was found to be extremely sensitive (with a quantitation limit of 0.25 ppm), reproducible and compatible with 1x PBS formulation chemistry. The two Lots of final vaccine product tested did not show any detectable levels of hydrazine impurity by this method. This enabled us to establish release criteria based on the DL (detection limit) calculated on this method.

Conclusions

The validated UPLC method described in this report, is an upgraded and modified version of a previously published HPLC method, and works for the microanalysis of hydrazine in a 1xPBS formulated aqueous liquid vaccine without interference. This method is more sensitive, faster (5 minutes run time) and reproducible.

Acknowledgements

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