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Quantification of residual EDU (*N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC) hydrolyzed urea derivative) and other residual by LC–MS/MS

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Abstract

An LC–MS/MS method for determination of the break down product of *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC) urea derivative, EDU, has been developed and validated for monitoring the residual coupling reagents. Results indicate that the method exhibits suitable specificity, sensitivity, precision, linearity and accuracy for quantification of residual EDU in the presence of meningococcal polysaccharide-diphtheria toxoid conjugate vaccine and other vaccine matrix compounds. The assay has been validated for a detection range of 10–100 ng/mL and then successfully transferred to quality control (QC) lab. This same method has also been applied to the determination of residual diaminohexane (DAH) in the presence of EDU. LC–MS/MS has proven to be useful as a quick and sensitive approach for simultaneous determination of multiple residual compounds in glycoconjugate vaccine samples.

Keywords: LC–MS; Quantification; Validation; Assay transfer; Polysaccharide protein conjugate vaccine; Residual material

1. Introduction

Covalent linkage of bacterial polysaccharide antigens to protein carriers confers a number of desirable properties to the final vaccine such as improved protection of infant recipients and T-dependent immunological memory [1–5]. For several major types of encapsulated organisms such as *Haemophilus influenzae* type b, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, the capsular polysaccharides do not always contain chemically reactive groups such as amino or carboxyl moieties that can be covalently linked directly to a protein carrier. A variety of methods have been developed for chemically coupling the polysaccharide and protein moieties in conjugate vaccines [1]. One approach involves activation of the polysaccharide carboxylic acid groups using *N*-ethyl-*N*′-(dimethylaminopropyl) carbodiimide (EDC) [6–8]. The activated polysaccharide can then be linked to the protein carrier directly. The application of EDC results in a number of stable, "peptide" linkages between the polysaccharide and protein as well as intra-chain ester linkages within the polysaccharide component. As a consequence of the reaction, EDC is converted to an inert by-product (*N*-ethyl-*N*′-(3-dimethylaminopropyl) urea, EDU). Unreacted EDC, EDU and other compounds such as diaminohexane (DAH, a spacer molecule bridge between protein and polysaccharide components) must be removed from the vaccine product during subsequent purification steps. Monitoring the residual level after removal of these compounds is important for assuring product purity and process consistency. Therefore, quantification and identification of residual compounds such as EDC, EDU and DAH are necessary.

Quantification of EDC by chromatographic methods is difficult due to poor UV absorbance at low levels [9].

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Furthermore, the currently used colorimetric method is specific to EDC and cannot quantify EDC that has been converted to EDU. Finally, UV detection has the general disadvantage that it cannot always identify detected peaks unambiguously. Kinetic studies [10] on the rate of hydrolysis of EDC to EDU in aqueous solutions demonstrates that, under commonly used conjugation conditions (pH ∼ 5), after 2 h reaction, more than 99% EDC is irreversibly converted to EDU. Therefore, monitoring of residual EDC has been replaced with monitoring of residual EDU after the completion of the conjugation and the purification process. Consequently, a separate test for the detection and quantification of EDU was developed. Mass spectrometry detection has the advantages of higher sensitivity and higher specificity [11–17]. Additionally, MS has the potential to measure several residual compounds in a single test with unambiguous assignments.

This paper describes the development, validation and transfer of an LC–MS/MS method for fast and specific detection of EDU. We have also observed that the method is capable of simultaneous detection of other potentially coexisting residual compound such as DAH although with lower sensitivity for these compounds due to the difference in ionization efficiencies for the two different types of compound. A complete validation and transfer study was carried out on quantification of EDU to confirm accuracy, precision, linearity, specificity and robustness of the method. Validation and transfer the assay of EDU measurement will be discussed in detail. Also included in this report, linearity, accuracy, precision and specificity were assessed for DAH, and it has the similar specs comparing to those factors measured for EDU.

2. Experimental

2.1. Chemicals and reagents

N-Ethyl-*N*′-(dimethylaminopropyl) carbodiimide hydrochloride, diaminohexane (DAH), *N*,*N*′-diisopropylcarbodiimide (DIC), and HPLC grade formic acid were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile was purchased from J.T. Baker. Purified water was prepared using a Milli-Q plus Ultra-Pure water system (Millipore, Bedford, MA, USA). Sodium chloride solution (0.85%, w/v) was supplied in house and this has been used as matrix to simulate the matrix solution of the conjugate product. All other chemicals and solvents were of analytical-reagent grade. Protein, polysaccharide, and protein-polysaccharide conjugates were obtained from the Aventis Pasteur Product Development Department.

EDC was prepared as a stock solution at 1 mg/mL in water containing 1% (v/v) formic acid (pH ∼ 2.2) to acidify the solution and to convert the starting EDC to EDU. The stock solution of EDC/EDU was prepared at least 24 h prior to use to ensure complete conversion of EDC to EDU. MS analysis has been used to verify that EDU is the hydrolysis product of EDC upon acidification [11]. Reagent DAH and internal standard DIC were also prepared at 1 mg/mL in 1% (v/v) aqueous formic acid solution. Under these conditions, DIC was quantitatively converted to the corresponding urea derivative DIU (data not shown). All these compounds were further diluted by sodium chloride solution (0.85%, w/v) to 1 μg/mL to be used for the preparation of reference standard.

2.2. Sample preparations

Stock solution for preparing reference standards (EDU and DAH), with DIU (*N*,*N*′-diisopropylcarbodiimide urea derivative) stock solution used as internal standard, were stored at 2–8 °C for up to 6 months. Concentration of 500 ng/mL of DIU was used as an internal standard for all samples. EDC reference samples were prepared in the range of 10–100 ng/mL (10, 20, 40, 60, 80, 100 ng/mL) and DAH were prepared in the range of 1–10 μg/mL (1, 2, 4, 6, 8, 10 μg/mL). EDU content was measured in each of the individual four serotypes of monovalent meningococcal polysaccharide–protein conjugate concentrates (serotypes A, C, W and Y). For the specificity measurements, a known amount of EDU was spiked into each serotype of monovalent conjugate vaccine (A, C, W and Y) to measure the recovery. Polysaccharide and protein used for conjugation without EDC treatment were also used to check the specificity, by the absence of EDU content in the matrix mixture when no EDC was added, and the recovery of known amount of spiked EDU after it is added. All samples, including stock reference materials, go through Centricon filtration. After filtration, retentate containing conjugates and all large size molecules were discarded. Filtrate containing the residual compounds was used for further analysis. Amicon Centricon concentrators (3 kDa cutoff) with 2 mL capacity were used to separate the small molecular weight material from the large MW material such as glycoconjugate, proteins and polysaccharide.

2.3. Chromatography

The HPLC system consisted of a Finnigan MAT (San Jose, CA) Spectra system P4000 pump, AS3500 autosampler. Samples were injected at 20 uL for analysis. Chromatographic separations were performed on a Zorbax (Agilent, Santa Clarita, CA) RX-C18 column (150 mm × 2.1 mm i.d.), operated at ambient temperature. The mobile phase, consisting of 0.1% (v/v) formic acid in water/acetonitrile (99/1, solvent A) and 0.1% (v/v) formic acid in water/acetonitrile (1/99, solvent B), was delivered at a flow rate of 0.2 mL/min with a linear increase of solvent B from 0 to 40% over 20 min. After a 5 min rinse at 50:50% of A and B, the system was equilibrated in buffer A for an additional 10 min. Each run required a total of 35 min. For the assay transfer, Jasco HPLC has been used.

2.4. Mass spectrometry

A LCQ^{duo} (Finnigan, San Jose, CA) with electrospray source was used. Heated capillary temperature was maintained at 200 °C. Ion optics were automatically tuned

at MW of 145.1 (DIU, the hydrolyzed DIC MW) and ESI source condition was optimized. Sample identification was performed for the EDC/EDU, DAH, and DIC/DIU prior to the quantification. Quantification was performed by MS/MS method using single ion monitoring (SIM) of three precursor—three product ion transitions at *m/z* 174.2 $[M+H]^+ \rightarrow 129.2$ (for EDU → fragment ion), *m/z* 117.2 $[M+H]^+ \rightarrow 100.2$ (for DAH → fragment ion), and *m/z* 145.1 $[M+H]^+ \rightarrow 60.1$ (for DIU → fragment ion). Twenty-five percent of collisional energy was applied to all three compounds. The peak widths of precursor ions were maintained at ∼0.7 u at half-height in the SIM mode.

Data acquisition, peak integration, and calculation were performed using $LC_{Quan}{}^{TM}$ software residing in the Xcalibar program. Peak area ratios of analytes to internal standards were utilized for the construction of calibration curves using equal weighted linear least-squares regression of compound concentrations and measured peak area ratio. The concentration of analytes in quality control or unknown samples was calculated by interpolation from the calibration curves.

MicroMass Quattro LC mass spectrometer was used for the assay-receiving lab. Same precursor to fragment ion detections for the EDU and DIU quantifications were setup in MRM mode, and Masslynx software was used for data analysis.

2.5. Validation procedure for quantification

Three QC samples at three concentration levels (samples prepared at 20, 40, 80 ng/mL) were used to assess the system suitability of each run. Accuracy, precision, and detection limit for standard reference at all levels and samples plus spikes were assessed from three independent runs against expected values and by percentage relative standard deviations. Specificity was assessed in two experiments: (1) different EDU levels (10–60 ng/mL) were spiked into the individual serotype monovalent conjugates (which are the target samples for EDU content measurement), and the recovery of the spiked material has been used to assess the specificity; (2) one level of known EDU amount spiked into the simulated matrix, prior to the EDC treatments, which has polysaccharide, protein and other matrix components in the mixture except EDC. This set of experiments is for the purpose of proving the absence of an EDU signal when there is no EDU present and accurate detection of EDU when it is spiked into the simulated matrix.

Intermediate precision/robustness was assessed by varying days, columns, operators, flow rate, electrospray voltage and filtration time. Linear least-square regression was used to assess the concentration response relationship of standard reference material and spiked samples. Assay range was determined to be 10–100 ng/mL with acceptable precision and accuracy. The assay was determined to be specific since no unaccounted fragment ions were detected. The validation experimental design matrix is shown in Fig. 1, with a reference standard in a range of 10–100 ng/mL, six levels of spiked material for all four serotypes, and robustness test designed matrix for each serotype. A Plackett–Burman design [18] was used to assess intermediate precision as shown in Fig. 1, with the four individual serotypes of monovalent meningococcal polysaccharide–diphtheria toxoid (Mn–Dt) conjugate concentrates. Seven factors from A to G were setup: two different operators (factor A), different days (B), two serial number C18 columns (C) two flow rates (D) (0.2 μL/min versus 0.22 μL/min), different filtration time (E) (3 h versus 3.5 h), two different electrospray voltages (F) (3.5 kV versus 4 kV). G is a null factor used in the evaluation matrix. Each serotype was evaluated according to the table in Fig. 1: (+) and (−) signs represent each one of the two conditions for each factor respectively as listed above.

Data were analyzed by calculating "%effects" (factors) and then ranking them and plotting on normal probability paper. Factors with a "%effects" calculated less than 10% were considered robust. Factors with "%effects" greater than 10% are not considered robust and need to be controlled during experimentation. The percentage relative standard deviation (%R.S.D.) for robust factors was calculated and used as an estimate of the precision of the method.

Reference Standard range: 10ng/mL, 20ng/mL, 40ng/mL, 60ng/mL, 80ng/mL, 100ng/mL

Spiking Samples: each serotype (A, C, W135 and Y) were spiked with 10 ng/ml, 20 ng/ml, 30ng/mL, 40ng/mL, 50ng/mL, and 60ng/mL

8 factor robustness test designed in a matrix (Intermediate Precision for each serotype)

	A	B	C	D	E	F	G
1	+	+	+	-	+	-	-
2	-	+	+	+	-	+	-
3	-	-	+	+	+	-	+
4	+	-	-	+	+	+	-
5	-	+	-	-	+	+	+
6	+	-	+	-	-	+	+
7	+	+	-	+	-	-	+
8	-	-	-	-	-	-	-

Factors
A: Operator
B: Day
C: Column
D: Flow rate
E: Filtration time
F: Voltage
G: z = Null

Fig. 1. Validation design matrix for EDU quantification.

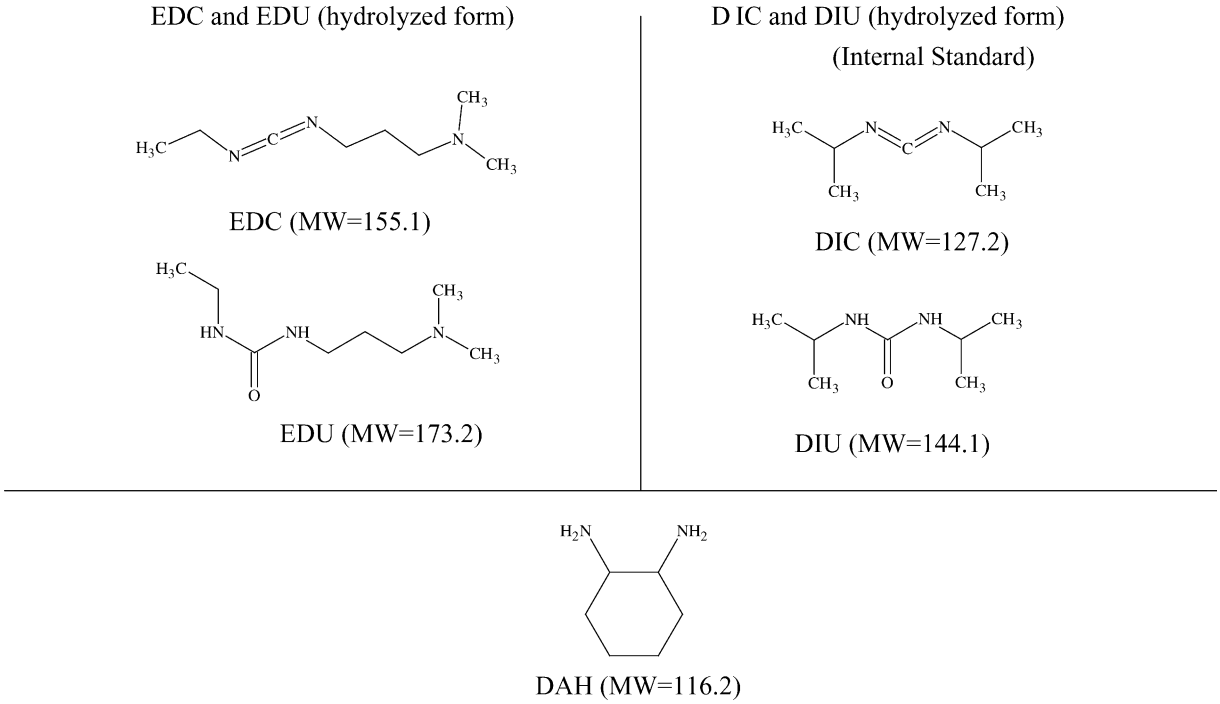

Fig. 2. Structures of EDC/EDU, DAH and internal standard DIC/DIU.

3. Results and discussions

3.1. MS and MS/MS analysis of the analyte

Experiments were run on individual samples for structure identification prior to the separation and quantification. Fig. 2 shows the structure of each chemical compound and its hydrolyzed form. Fig. 3a–c shows the full mass range scan of each molecule (measuring their stock solution by direct infusion) on top and their CID spectra on the bottom with the collisional energy set at 25% for structure identifications.

Only hydrolyzed urea derivatives (ions $[M+H]^+$ at 174.2 Da for EDU and at 145.1 Da for DIU) were observed in the full scan of EDC/EDU (Fig. 3a) and DIC/DIU samples (Fig. 3b). Non-hydrolyzed starting material forms (ions $[M+H]^+$ at 156.1 Da for starting material of EDC and at 128.2 Da for starting material of DIC) are not observable in the full scan (with a sensitivity of 10 ng/mL) (Fig. 3a and b), as well as the analysis of the conjugate sample (serotype A filtrate with loop injection, see Fig. 3d). Kinetic studies carried out by MS and CE measurements referenced in [10] indicate the complete conversion of the EDC to EDU. Conversion of EDC to EDU has also been confirmed using a separate colorimetric assay in which EDC can be directly quantified (detection limit of 1 μg/mL for EDC, after the conjugation process (data not shown). Therefore, the goal of monitoring EDC residuals during the process has been changed to the monitoring of EDU in the conjugate matrix sample. Quantification of EDU is done by MS–MS by monitoring molecular weight at 174.2 → 129.2 Da, and the internal standard DIU quantification was set to 145.1 → 60.1 Da. Unreacted DAH is stable during the conjugation process (data not shown), therefore the quantification of the residual DAH was also done by MS–MS by monitoring the molecular weight at 117.2 → 100.2 Da (Fig. 3c).

LC–MS/MS was setup for monitoring of the two residual components (EDU and DAH) with DIU MS/MS (145.1 → 60.1 Da) content as the internal standard. A single run takes 35 min, with DAH, EDU and DIU detected at 3.5, 7.1 and 13.9 min. Total ion chromatogram (TIC) for each run is presented in Fig. 4. Sensitivity of detection for DAH was lower than that of EDU in the same ESI setup by about 100-fold. The loss of sensitivity for DAH might be due to the difference in the ionization efficiency.

3.2. Quantification—calibration curve: linearity, precision and accuracy

The assay was developed and demonstrated the simultaneous detection of EDU and DAH. Linearity, precision, and accuracy have also been checked, and validation was then continued focusing on the EDU. Linear calibration curves for EDU and DAH were obtained over the range of 10–100 ng/mL and 1–10 μg/mL, respectively, by plotting concentration versus the peak area ratio of the respective analyte and the internal standard. Fig. 5a shows an overlay of the calibration curve for EDU at 10–100 ng/mL ranges along with the linear regressions of $r^2 = 0.9980$ and $y = 1.0x + 0.1$. Similarly, the linearity analysis of DAH yield an $r^2 = 0.9908$,

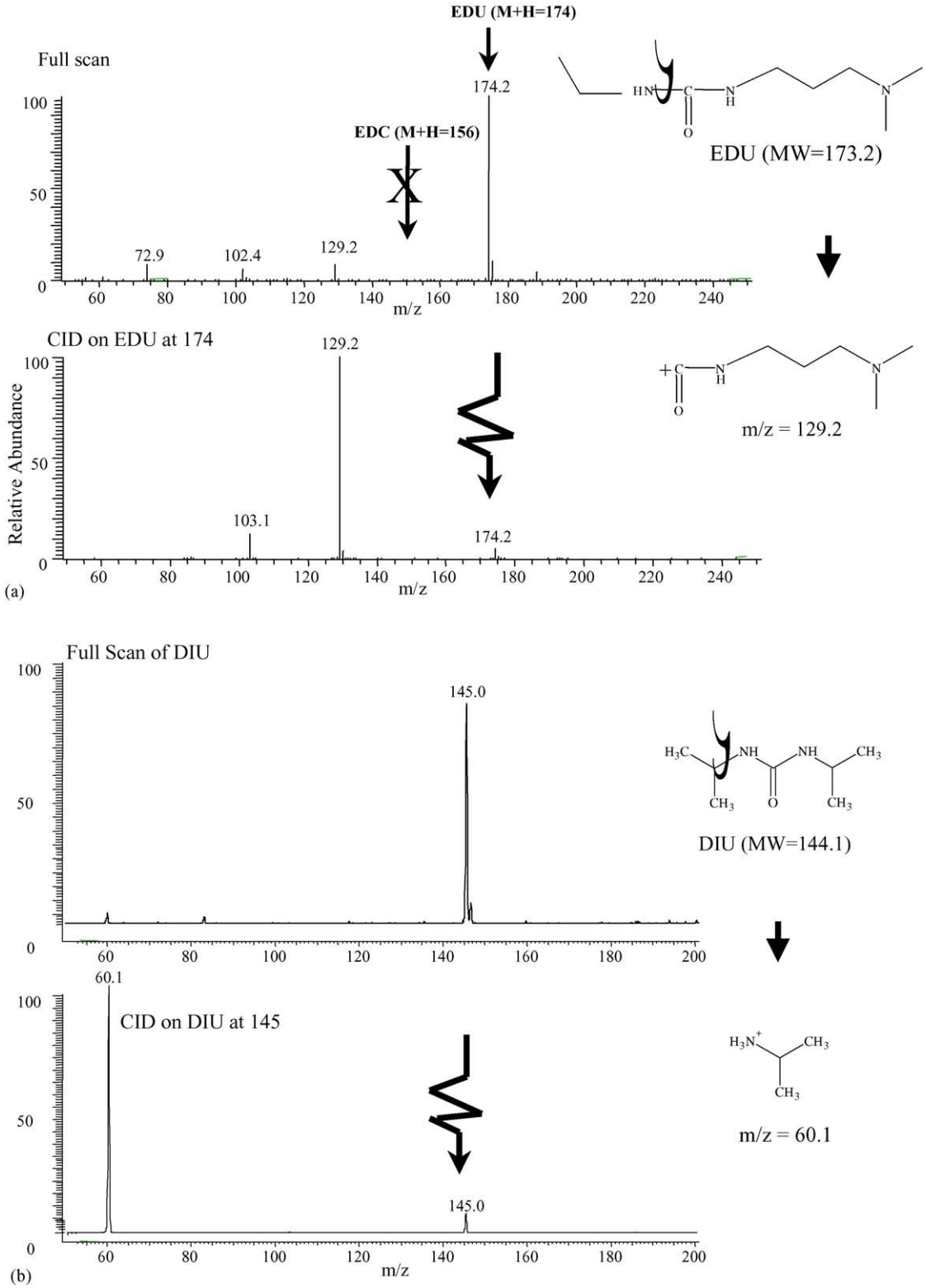

Fig. 3. MS and MS/MS spectra for structure and compound identification of (a) EDC/EDU; (b) DIC/DIU; (c) DAH; and (d) full scan (60–200 *m*/z) of meningococcal conjugate vaccine serotype A filtrate.

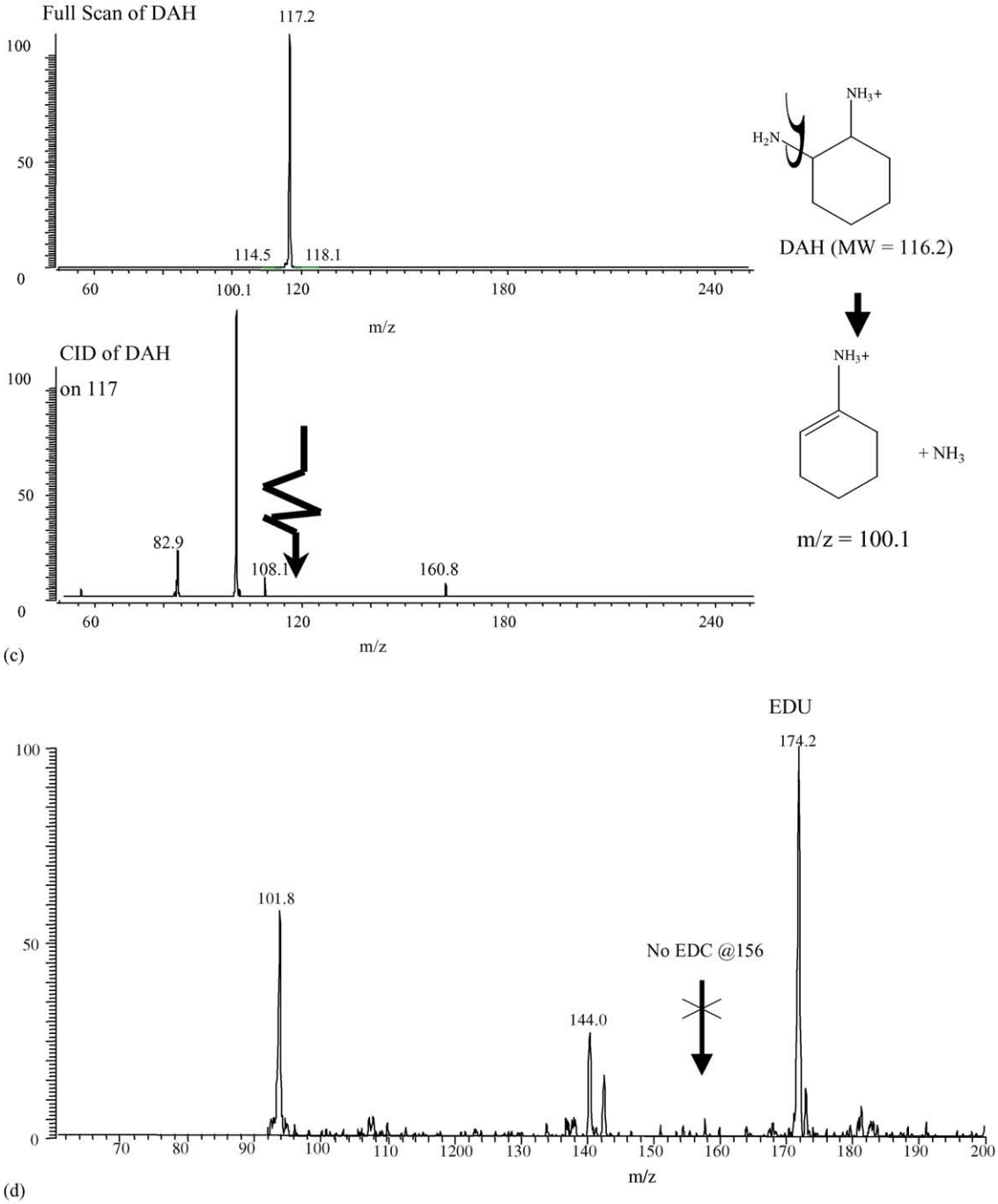

Fig. 3. (*Continued*).

and $y = 0.7x + 0.1$. The correlation coefficients (r^2) of the calibration curves were ≥0.99. Results of reference standard EDU and DAH are summarized in Table 1a and b, respectively. Precision for three independent measurements of each level was assessed at a %R.S.D. ≤5%. Accuracy of quantification measurement was calculated by %bias = [(mean measured value − theoretical value)/theoretical value] × 100 and shown to be ≤7%, absolute value.

3.3. Quantification—range of the measurements and the limit of quantitation

For the analysis of reference standard curve data in the range of 10–100 ng/mL, measurement accuracy (|%bias| < 4), linearity ($r^2 > 0.99$), and precision (%R.S.D. < 5%) met the study acceptance criteria. Therefore, 10–100 ng/mL can be set for the EDU measurement range.

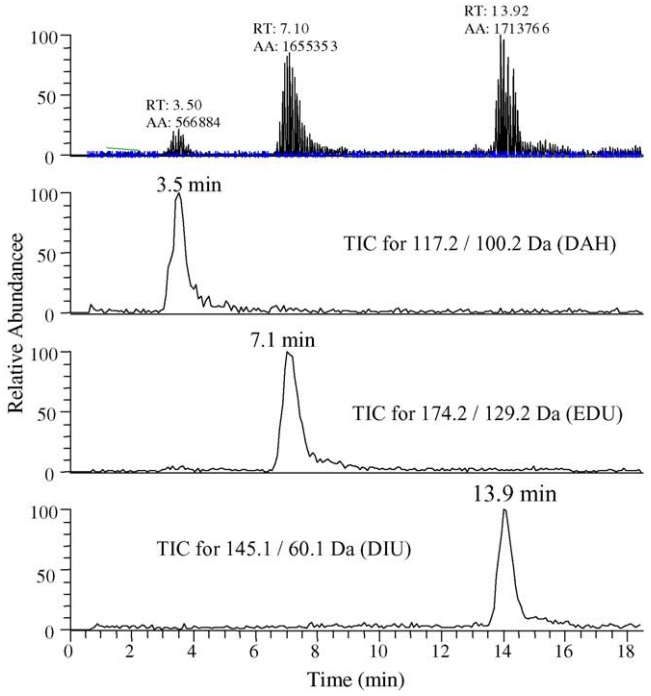

Fig. 4. Ion chromatogram for LC–MS/MS detection of DAH, EDU and DIU (internal standard).

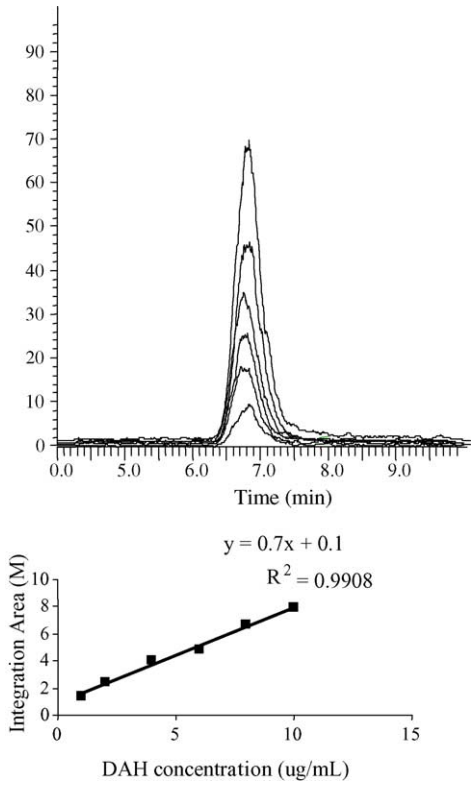

Fig. 5. (a) Ion monitoring profile for EDU at 10–100 ng/mL levels. (b) Linear curve for DAH at 1–10 μg/mL levels.

Table 1
Linearity of reference curve for (a) EDU; (b) DAH

Std conc.	AVE	STD	%STD	%Bias
(a)				
10 ng/mL	9.8	0.5	5	−2
20 ng/mL	20.0	0.8	4	0
40 ng/mL	40.5	0.2	1	1
60 ng/mL	61.6	1.5	2	3
80 ng/mL	77.1	2.4	3	−4
100 ng/mL	101.0	3.9	4	1
(b)				
1.0 μg/mL	1.0	0.026	2.6	0.4
2.0 μg/mL	2.1	0.023	1.1	−3.5
4.0 μg/mL	3.9	0.067	1.7	2.2
6.0 μg/mL	5.6	0.065	1.1	6.0
8.0 μg/mL	8.5	0.026	0.3	−6.4
10.0 μg/mL	9.9	0.105	1.1	1.3

3.4. Quantification—linearity, precision and accuracy for the samples and spiking materials in the detection range, and specificity

Table 2a summarizes EDU measurement results from the triplicate runs for meningococcal monovalent conjugate vaccine samples serotypes A, C, W-135, and Y at six different levels of spikings with EDU (10–60 ng/mL). Spiked data were assessed by linear regression. For all four serogroups: $r^2 = 0.9983$ for group A; $r^2 = 0.9957$ for MnC; $r^2 = 0.9934$ for MnW-135; and $r^2 = 0.9986$ for MnY. With 10 ng/mL increments of EDU spiked into each of the four conjugate serotypes, the measured values increased accordingly, with a %R.S.D. that ranged from 1 to 4% for type A, 1 to 9% for type C, 1 to 9% for type W-135, and 2 to 15% for type Y. Accuracy was assessed by calculating %bias, where %bias = [[(spiked sample value − unspiked sample value) − amount spiked]/amount spiked] × 100. Percent bias ranged from 6 to 12% for type A, 3 to 14% for type C, 0 to 9% for type W-135 and, 0 to 5% for type Y. Spiking study data were analyzed by least-squares linear regression. The r^2 values for each of the curves generated for the spiked serotype samples were greater than 0.99 (Fig. 6).

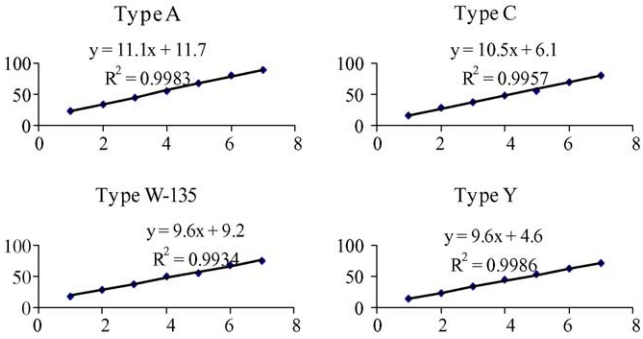

Fig. 6. Linearity measurements in the detection range for four serotypes of samples with spiking materials at six different spiking levels of EDU [in each case, the *x*-axis represents each serotype EDU level and the spikes (number of points); and the *y*-axis represents EDU contents from MS measurements (see also Table 2)].

Table 2
(a) Precision and accuracy of four serotypes of conjugate samples and these samples with spiking EDU at six different levels (ng/mL); (b) specificity measurements on the EDU spiked samples at 50 ng/mL levels and non-spiked matrix samples

Type A	Mean RV	%Std	%Bias	Type C	Mean RV	%Std	%Bias
(a)							
A	23.5	3	–	C	16.7	4	–
A + 10	34.0	4	6	C + 10	28.1	8	14
A + 20	44.7	4	6	C + 20	37.4	9	4
A + 30	54.5	3	3	C + 30	48.9	4	8
A + 40	68.4	2	12	C + 40	55.6	5	−3
A + 50	79.5	1	12	C + 50	70.0	4	7
A + 60	89.3	1	10	C + 60	81.1	1	7
Type W	Mean RV	%Std	%Bias	Type Y	Mean RV	%Std	%Bias
W	17.9	9	–	Y	13.4	15	–
W + 10	28.2	4	2	Y + 10	23.9	7	5
W + 20	38.1	5	1	Y + 20	33.5	6	0
W + 30	50.6	3	9	Y + 30	44.6	3	4
C + 40	54.8	1	−8	Y + 40	52.7	6	−2
W + 50	67.7	7	0	Y + 50	62.0	4	−3
W + 60	75.5	5	−4	Y + 60	71.2	2	−4

Sample	EDU content measured (ng/mL)	%Recovery
(b)		
Matrix A + 50 ng/mL	46.9	94
Matrix A	N/F	
Matrix C + 50 ng/mL	47.5	95
Matrix C	N/F	
Matrix W + 50 ng/mL	51.4	103
Matrix W	N/F	
Matrix Y + 50 ng/mL	48.2	96
Matrix Y	N/F	

N/F indicates peak not being found.

Table 2a also *implies* the specificity of this method. Spiked EDU can be accurately recovered and measured in the sample matrix indicates the method measures what it intents to do. Since the conjugate samples alone have EDU content in the matrix (not completely removed during the wash process after conjugation), a second set of samples with no known EDU was prepared and spiking experiments were run. Table 2b summarizes the second set of experiments designed to demonstrate specificity. The four serotype matrix samples (polysaccharide, protein, and other possible residuals such as salt and hydrazide, etc.) that have not been treated by EDC/EDU previously were chosen. Comparison between matrix samples alone and the EDU spiked matrix samples were analyzed. Data presented in Table 2b show that this method is specific for EDU measurements. No EDU was detected in the matrix samples alone, and EDU spiked at 50 ng/mL was recovered in a range of 90–110%.

3.5. Quantification—intermediate precision and robustness

Four individual serotypes of meningococcal monovalent conjugate concentrate vaccine samples were analyzed with two serial number C18 columns from same vendor, with two different operators, different days, two flow rates (0.20 μL/min versus 0.22 μL/min), different filtration time (3 h versus 3.5 h), and two different electrospray voltages (3.5 kV versus 4 kV). Robustness analysis on each serotype has been evaluated according to Table 3-1 and robustness analysis guideline in [11]. Ranked effect of each type has been summarized in Table 3 (2a–d). For all four serotype monovalent concentrate vaccines A, C, W and Y, % ranked effects for all factors are all smaller than 9%, and their S.D., R.S.D. and upper 95% CI of the S.D. were calculated. The results indicate that these factors are not considered statistically significantly different.

3.6. Assay transfer evaluation—precisions and accuracies

Table 4a summarizes the calculated precision results for each of the four monovalent serogroups that were analyzed by component variance analysis using SAS® for experiments carried out in two labs (development lab and assay-receiving lab). All runs satisfy the acceptance criteria for precision with population CV's less than 20%. Table 4b summarizes the calculated accuracy results for each or the four monovalent conjugate serogroups. Accuracy is assessed by calculating the ratio of observed (QC lab) to expected results (development lab). All serogroups pass the acceptance criteria for accuracy with relative accuracy between 85 and 115%. Based on the

Table 3
Ranked effect (1) measurement setup for intermediate precision; (2a) for the serotype A sample; (2b) for the serotype C sample; (2c) for the type W-135 sample; (2d) for the type Y sample

Factors			*M*-values
(1)			
Operator			−1.35
Day			−0.76
Column			−0.35
Flow rate			0
Filtration time			0.35
Voltage			0.76
z			1.35
Factors	Ranked effects	*M*-values	%Effect
(2a)			
Flow rate	−0.21	−1.35	−1
Filtration time	−0.09	−0.76	0
Null	−0.08	−0.35	0
Day	0.54	0	2
Operator	0.87	0.35	2
Column	0.99	0.76	3
Voltage	1.76	1.35	5
Mean: 19.9	S.D.: 1.2	R.S.D.: 6%	95% CI: 0.8
(2b)			
Null	0.16	−1.35	1
Flow rate	0.38	−0.76	1
Column	0.88	−0.35	3
Operator	1.09	0	4
Day	1.19	0.35	4
Filtration time	1.76	0.76	6
Voltage	2.24	1.35	7
Mean: 15.7	S.D.: 1.8	R.S.D.: 10%	95% CI: 1.2
(2c)			
Null	−1.05	−1.35	−3
Flow rate	−0.23	−0.76	−1
Operator	−0.09	−0.35	0
Day	0.61	0	2
Voltage	1.23	0.35	3
Filtration time	1.67	0.76	4
Column	2.78	1.35	7
Mean: 20.5	S.D.: 12.0	R.S.D.: 10%	95% CI: 1.4
(2d)			
Day	−1.11	−1.35	−4
Operator	−0.87	−0.76	−3
Flow rate	−0.34	−0.35	−1
Null	−0.19	0	−1
Filtration time	−0.07	0.35	0
Column	1.68	0.76	6
Voltage	1.76	1.35	6
Mean: 14.9	S.D.: 1.5	R.S.D.: 10%	95% CI: 1.0

Ranked effect for factor is the sum of test results based on sign of factor divided by 8, they will be put in the table in an ascent order; *M*-values are the means of the order from statistics for a sample size of seven and they are the fixed value as listed below. Orders of the factors can be different from the table according to the calculated ranked effect.

analysis of the data obtained, it is concluded that the precision and accuracy for this assay being transferred were met for all four monovalent concentrates, and the QC laboratory is qualified to perform the assay.

Table 4
Summary of the calculated (a) precision results for each of the four monovalent serogroups that were analyzed by component variance analysis using SAS® for experiments carried out in two labs (development lab and assay-receiving lab); (b) accuracy results for each or the four monovalent conjugate serogroups

Sample type	CV population (%) as per SAS®
(a)	
Type A (development lab)	6.19
Type C (development lab)	2.71
Type Y (development lab)	11.35
Type W135 (development lab)	1.79
Type A (QC lab)	2.83
Type C (QC lab)	14.63
Type Y (QC lab)	15.56
Type W135 (QC lab)	7.63
Sample type	Relative error (%)
(4b)	
Type A	103.7
Type C	102.9
Type Y	103.3
Type W135	94.75

Accuracy is assessed by calculating the ratio of observed (QC) to expected results (development lab).

4. Conclusions

LC–MS/MS with pre-sample treatment has been successfully applied to monitor the residual level of EDC derivatives (at 10–100 ng/mL) with simultaneous detection of DAH (at 1–10 μg/mL). MS can therefore be used to detect and quantify these compounds with greater sensitivity, precision and specificity than currently published methods, even though DAH has a lower ionization efficiency compared to EDU and DIU. Assay validation and transfer on EDU quantification have been successfully achieved in development lab and QC testing lab. This method should be applicable for multivalent conjugate vaccine formulations and has the potential for multi-residual analysis.

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