Development and application of HPLC-RI and HPLC-MS/MS based methods for quantification of residual deoxycholate levels in pneumococcal polysaccharides

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A B S T R A C T
The analysis of residual sodium deoxycholate (DOC), a detergent of biological origin used in manufacturing of polysaccharide vaccines is challenging due to complex sample matrices and the lack of suitable methods. Here we report, rapid and sensitive high-performance liquid chromatography-refractive index (HPLC-RI) and tandem mass spectrometry (HPLC-MS/MS) methods for estimation of residual DOC in pneumococcal polysaccharides. For HPLC-RI method, separation was achieved using Luna C18 column and mobile phase compositions of acetonitrile: methanol and water (0.1% formic acid). MS/MS method showed linearity (r² = 0.997) over the range of 10–320 ng/mL with limits of detection (LOD) and lower limit of quantitation (LOQ) of 3 and 10 ng/mL respectively. Precision (% RSD) and accuracy (% recovery) for both methods were in the range of 0.74–8.29% and 82.33–117.86% respectively. Sample matrices interferences were addressed following novel sample clean-up method based on liquid–liquid extraction. Both methods enabled traceable quantitation of DOC in intermediate and purified pneumococcal polysaccharides of serotypes: 1, 5, 6A, 6B, 7F, 9V, 14, 19A, 19F and 23F.

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1. Introduction

Polysaccharide vaccines such as against Neisseria meningitidis, Streptococcus pneumoniae, Haemophilus influenzae type b, and Salmonella typhi, induces protective host immune responses against specific capsular polysaccharides. The manufacturing processes of such vaccines involve basic steps comprising of a) preparing a fermentation culture of bacterial cells that produce capsular polysaccharides b) lysing the bacterial cells in said fermentation culture and c) isolation of capsular polysaccharides from the lysed culture. The lysis of the cells is an important step at the end of the fermentation and is accomplished by the use of surfactants, such as deoxycholate (sodium salt of deoxycholic acid; DOC) or similar congeners. DOC is reportedly to be the most preferred surfactant for vaccines including, meningococcal, tuberculosis, pneumococcal, typhoid, etc [1–4]. Chemically, DOC represents a steroidal detergent with polar hydroxyl and non-polar methyl groups on the surface, wherein polar groups form an interface with aqueous media and non-polar groups self-associate to form micelles. These micelles further partitions into the membrane lipid layer leading to the lysis and release of cellular components. DOC is preferred in manufacturing processes, as it is a mild detergent, precipitation does not impact the antigenic characters of polysaccharide and most importantly can be easily removed using simple dialysis or chromatography based procedures [5]. The amount of DOC to be used in a particular process needs to be optimized and validated with respect to inactivation of bacteria, precipitation of proteins and removal during downstream processing. Additionally, there are regulatory requirements for monitoring and quantification of residuals of biological origin such as DOC in vaccines so as to minimize the safety risks to humans [6–8]. The residual analysis and monitoring of residual DOC levels during manufacturing of polysaccharide-based vaccines is challenging due to complex sample matrices and lack of suitable methods.

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Only two methods are published previously for determination of DOC in vaccines like samples and each has several drawbacks: Method 1: Szalkowski et al. reports an indirect colorimetric method for estimation of DOC in ox bile and was adapted by Girard et al. for determination DOC in vaccine matrices [9]. This method being colorimetric and indirect has limitations of sensitivity; specificity and most importantly require higher sample volumes. Method 2: Girard et al. reported HPLC-UV method for estimation of DOC in influenza vaccine, which requires large sample quantity for solid phase extraction for sample clean up. Additionally, UV based detection has limitation with respect to sensitivity as DOC has inherent low absorptivity in the region of 200–210 nm [10]. There are reports on other methods using evaporative light scattering detection (ELSD), mass spectrometry (MS) detection for determination of free and conjugated bile acids including DOC in human faeces, serum and bile samples [11–13]. However, a direct adaptation of such methods to vaccine matrices is difficult since these methods are designed to estimate free and conjugated bile acids in biological matrices and necessitate steps such as fractionation, concentration, and derivatization. Hence, there is an urgent need in the prior art for rapid, highly specific and sensitive methods for quantification of DOC residues in vaccine samples. We report here development and validation of two new rapid and sensitive methods based on HPLC-RI and HPLC-MS/MS for determination of DOC in purified and intermediate pneumococcal polysaccharide samples. The study suggests that both methods were rapid, reproducible and met all the validation criteria. HPLC-MS/MS was identified as the method of choice for process monitoring and control of this residual, as it allowed higher sensitivity to 10 ng/mL levels in complex intermediate pneumococcal polysaccharide samples. The study also suggests an important role of sample preparation in achieving the required accuracy.

2. Materials and methods

2.1. Chemicals and solvents

Sodium deoxycholate (DOC) standard (% purity ≥ 97) was purchased from Sigma–Aldrich (Mumbai, India). Methanol, acetonitrile, formic acid and acetic acid (HPLC grade) were procured from Rankem (Mumbai, India). Sodium acetate was procured from Sigma–Aldrich (Mumbai, India). HPLC grade water was used from J. T. Baker (Mumbai, India).

2.2. Standard preparation

Stock of standard solution of 1 mg/mL was prepared by diluting 10 mg of DOC in 10 mL of methanol. Working stock of 0.1 mg/mL was prepared in methanol from the above stock and subsequently used for further studies.

2.3. Pneumococcal polysaccharide samples

Pneumococcal polysaccharide samples manufactured at Serum Institute of India Pvt. Ltd, Pune, India was used for analysis. Intermediate and final purified samples from serotypes such as 1, 5, 6A, 6B, 7F, 9V, 14, 19A, 19F and 23F were analyzed for the DOC levels using HPLC-RI and HPLC-MS/MS methods.

2.4. Extraction of DOC from samples: spin filtration approach

500 μL of polysaccharide samples were loaded to 10 kDa centrifugal filter (Millipore, Ultracef® 10 K) and centrifuged at 7798 × g for 15 min. The filtrate was collected and 50 μL was used for HPLC-RI analysis.

2.5. Extraction of DOC from samples: liquid–liquid extraction method

200 μL of acidified acetonitrile (0.4% v/v of acetic acid) was added to 200 μL of polysaccharide samples. The resulting solution was vortexed for 1–2 min and 400 μL of chloroform was added to this solution and again vortexed vigorously for 1–2 min for clear separation of organic and aqueous layers. The organic layer (lower layer) was collected and dried under stream of nitrogen at +40 °C and the resulting residue was reconstituted with 200 μL of mobile phase for the analysis. Injection volumes of 50 and 30 μL were used for HPLC-RI and HPLC-MS/MS analysis respectively.

2.6. HPLC-RI analysis

HPLC system of Water Alliance coupled with RI detector (2414) and photo-diode array detector (2996) was used for the analysis. Chromatographic separation of DOC was carried out using Phenomenex C18 column (250 mm × 4.6 mm, 5 μm particle size) with C18 guard column (Phenomenex) using isocratic elution of acetonitrile: methanol: 20 mM sodium acetate (pH 4.3 with acetic acid) in the proportion of 60:05:35 at flow rate of 1 mL/min. Column and detector temperature was kept at +35 °C. Autosampler temperature and RI detector sensitivity was pre-set at +4 °C and 512 MV respectively and total run time of 15 min. Data acquired and was processed using Empower 3 software (Waters Corporation, USA).

2.7. HPLC-MS/MS analysis

DOC analysis was performed using HPLC-MS system equipped with Agilent 1200 Quaternary Pump (G1312B), and a degasser (G1379B) (Agilent, Waldbronn, Germany) connected to an HTC Pal autosampler (CTC Analytics, Zwingen, CH). A Triple quadrupole mass spectrometer API 3200 (AB Sciex Instruments, USA) equipped with Turbo ion spray operating in negative electrospray ionization (ESI) mode was used. Gradient chromatographic separation of DOC was performed on a BDS Hypersil C18 column (100 mm × 4.6 mm, 5 μm particle size) procured from Thermo Scientific, India with guard column of BDS Hypersil C18 column (100 mm × 4.6 mm, 5 μm particle size). Mobile phase A was methanol and mobile phase B was 0.1% formic acid in water was used. Gradient elution was performed with 10% A for 1.0 min, a linear increase to 90% A until 1.1 min, followed by 90% A until 5.0 min and re-equilibration from 5.1 to 8.0 min with 10% A. Flow rate was set to 1.5 mL/min with injection volume of 30 μL. Turbo Ion Spray source was operated in the negative ion mode using the following settings: ion spray voltage = −4500 V, ion source heater temperature = −450 °C, source gas 1 = 40 psi, source gas 2 = 35 psi and curtain gas setting = 20 psi. Declustering potential, entrance potential and collision energy were optimized at −85, −2 and −26 V respectively. DOC was monitored in the selected pneumococcal polysaccharide samples using pseudo multiple reaction monitoring (MRM) mode at m/z of 391.24 > 391.24 with dwell time of 400 msec. Data was acquired and processed using Analyst 1.4.2 software (AB Sciex, USA).

2.8. Method validation

Methods were validated for parameters such as specificity, linearity, Limit of detection (LOD) and limit of quantitation (LOQ), precision and accuracy in accordance with ICH requirements on method validation [14].
2.8.1. Linearity, LOD and LOQ
Linearity was determined by using peak area versus concentration curves in triplicates. Concentration levels from 2 to 80 μg/mL and 10–320 ng/mL was evaluated for linearity for HPLC-RI and HPLC-MS/MS method respectively. LOD and LOQ were established on the basis of signal to noise (S/N) ratio of 3 and 10 respectively.

2.8.2. Accuracy and Precision
Spike recovery method was used for assessment for accuracy. Recoveries were established at three different levels in triplicates; lower level (LQC), middle level (MQC) and at higher level (HQC) covering the calibration curve range. HPLC-RI method accuracy was studied at 4, 16 and 64 μg/mL. For HPLC-MS/MS based method, levels at 20, 80 and 160 ng/mL were used for assessment. Percent recovery was calculated using following formula.

\[ \text{Percent recovery} = \frac{C}{A + B} \times 100 \]

where, C, A and B denotes concentration of spiked sample; spiked amount of DOC and amount of DOC present in sample respectively.

Precision (repeatability and intermediate precision) of both methods was studied using the above three QC samples; (LQC, MQC and HQC). For repeatability, samples were processed in accordance with liquid–liquid extraction described in Section 2.5 and all these samples were analyzed in five triplicates for DOC contents. For intermediate precision, samples were prepared and processed on three consecutive days and analyzed for DOC content in five replicates. The % RSD of DOC content was considered as measure of precision.

2.9. Method uncertainty

Methods uncertainty for estimation of DOC in polysaccharides was determined using standard published procedures [15–17].

Following Equation (1) was used for the determination of expanded uncertainty.

\[ U_{\text{expanded}} = k \sqrt{U_{\text{calibration}}^2 + U_{\text{precision}}^2 + U_{\text{accuracy}}^2} \]  

where \( U_{\text{expanded}} \) is expanded uncertainty and \( k \) is the coverage factor (for confidence interval 95%, \( k = 2 \)). The individual sources of uncertainty mentioned in Equation (1) were determined using following equations.

2.9.1. Uncertainty associated to the calibration curve
The value \( U_{\text{calibration}} \) is calculated using following equation

\[ U_{\text{calibration}} = \frac{S_{xx}}{S_{x0}} \]  

wherein term \( S_{xx} \) is the concentration calculated from the calibration curve and \( S_{x0} \) is the standard deviation of the concentration obtained from calibration curve using following equation.

\[ S_{xx} = \frac{S(r)(1 - \frac{1}{N + n})}{m^2 \sum_{i=1}^{n}(x_i - \bar{x})^2} \]

\[ S(r) = \sqrt{\sum (y_i - mx_i - b)^2 / n - 2} \]

where \( m \) is the slope of the line, \( b \) is the y-intercept of the line, \( N \) is the number of replicate unknowns, \( n \) is the number of the standards, \( \bar{y}_0 \) is the mean of \( N \) repeat measurements of \( y \) for the sample, \( \bar{y} \) is the mean of the \( y \) values for the calibration standards, \( x_i \) are the concentrations of the standards and “x-bar” is the average concentration of the standards.

2.9.2. Uncertainty associated to precision
The value is calculated using following equation

\[ U_{\text{precision}} = \frac{S}{x_0 \sqrt{n}} \]

where \( S \) is the standard deviation of the experimental data for precision and \( n \) is the number of assays.

2.9.3. Uncertainty associated to accuracy
This parameter is calculated using following equation

\[ U_{\text{accuracy}} = \frac{S(n)}{\sqrt{n}} \]

where, \( S(n) \) is the relative standard deviation of the recovery and \( n \) is the number of assays.

3. Results

We report here development and validation of HPLC based quantification of sodium deoxycholic acid (DOC) in pneumococcal polysaccharide samples using refractive index (HPLC-RI) and tandem mass spectroscopy (HPLC-MS/MS). Manufacturing of pneumococcal vaccine involves fermentation, purification and conjugation of multiple serotypes individually. Thus, analytical approach for testing of residual DOC levels was envisaged which should be applicable to all the serotypes and have adequate sensitivity to monitor and quantity residual amounts in purified samples. RI and MS modes of detection were selected based on their universality and selectivity. The methods were developed and qualified using capsular polysaccharides of serotype 1, 5, 9V and 23F as these serotypes represents relatively complex chemical composition (e.g. serotype 1, 5 and 9V), large molecular size (e.g. serotype 23F) and ionic nature (e.g. serotype 1 as zwitterionic species) [18]. Subsequently, methods were also applied to other serotypes.

3.1. Optimization of chromatographic conditions: HPLC-RI based method

DOC is a weak acid (pKa ≈ 6.0) and remains non-ionized form under acidic conditions (pH ≈ 4.5). Optimized separation was achieved on Luna C-18 column (maintained at 25 °C) and isocratic elution with a mobile phase consisting of sodium acetate buffer and acetonitrile. Several compositions of sodium acetate buffer and acetonitrile were studied. 20 mM acetate buffer (pH 4.3 using acetic acid) and acetonitrile (40: 60% v/v) resulted in the good chromatographic separation of DOC. Separation was further optimized using methanol (5% v/v) as a modifier in the mobile phase resulting in improved peak shape and stable baseline. Representative HPLC-RI chromatograms of DOC reference standard and DOC spiked polysaccharide samples are shown in Fig. 1. DOC peak was observed at the retention time (\( t_R \)) of 9.78 min (Fig. 1; chromatograms- B, D & E). RI detection of DOC was studied at adjustable attenuator range from 16 to 512 MV. Optimum results were achieved at 512 MV and the response was set at standard and with positive polarity. A significant improvement in method sensitivity was observed and amounts as low as 2 μg/mL of DOC was suitably detected and quantified (Fig. 1; chromatogram D).

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3.2. Sample preparation: extraction of deoxycholate from complex polysaccharide samples

Proteins, nucleic acid and crude polysaccharides are the most likely constituents of deoxycholate lysates during the manufacturing process. Therefore, sample clean-up and extraction steps were envisaged for accurate and precise quantification of DOC in intermediate and final samples of pneumococcal polysaccharides. Filter cartridges with 10 kDa cutoff were employed for sample clean up. Both samples and standards were passed through the disposable 10 kDa spin filters and elutes were collected and tested for DOC using HPLC-RI method. This method showed inconsistent recoveries of DOC in spiked samples. Attempts were made to further improve the recovery of DOC by using different centrifugation speeds and elution solvents, but no significant improvement was observed especially with large molecular size polysaccharides such as 23F serotype (Table 1). Additionally, HPLC-photodiode array detection of the eluates from as such samples and spiked samples (2 mg/mL) showed the presence of interfering peak at the retention time (tR) of 9.76 min. The interfering peak showed spectral wavelengths (λ) of 201.6, 220 and 276.0 nm suggesting the proteinaceous nature of the interfering peak.

Liquid–liquid extractions are the most widely used sample clean-up procedures for complex matrices. Intermediate samples of polysaccharides comprise of components with mixed polarities, therefore, multi-solvent extraction method was explored for the study. Multi-solvent–liquid extractions using chloroform or ethyl acetate with acidified acetonitrile as a modifier were studied. Use of chloroform with acetonitrile (0.4% v/v acetic acid) showed consistent recoveries for all the serotypes. Acetic acid played an important role in the improved partitioning of DOC in the organic layer and acetonitrile resulted in complete precipitation of protein impurities resulting in improved recoveries (Table 1). This was evident by the complete absence of interfering or co-eluting peaks at retention time of DOC which was observed with 10 kDa filter based method (Fig. 2; chromatograms D & E). A single extraction was found sufficient for complete recovery of DOC. Additionally, the reconstituted sample solution was found stable up to 24 h, when stored at 2–8 °C.

3.3. Optimization of MS and chromatographic condition

For MS/MS-based analysis, DOC was initially studied for the selection of parent ions in both the negative and positive ionization modes as shown in Fig. 3 A and B respectively. In negative ionization mode, ESI-MS of DOC shows the presence of ions at m/z 391.4 [M−H]−. Whereas, ESI-MS of DOC in positive mode resulted in the ions at m/z 415.3 [M+Na]+ suggesting the formation of sodium adduct (Fig. 3B). ESI-MS response in negative ionization mode (ion intensities at 7.4 e + 6 cps) showed significantly higher responses as compared to positive mode (ion intensities at 3.8 e + 5 cps). Therefore, ESI-MS in negative ionization mode was selected for the DOC analysis. Additionally, possibilities of

### Table 1

Comparative % recoveries of DOC using spin filtration and liquid–liquid extraction approaches.

<table>
<thead>
<tr>
<th>Sample</th>
<th>10 kDa spin filtration approach</th>
<th>Liquid–liquid extraction approach</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LQC level</td>
<td>MQC level</td>
</tr>
<tr>
<td>Serotype 1</td>
<td>73.04 (20.12)</td>
<td>85.48 (18.16)</td>
</tr>
<tr>
<td>Serotype 5</td>
<td>78.25 (21.93)</td>
<td>82.95 (17.85)</td>
</tr>
<tr>
<td>Serotype 9V</td>
<td>76.45 (14.82)</td>
<td>87.89 (12.68)</td>
</tr>
<tr>
<td>Serotype 23F</td>
<td>65.98 (24.23)</td>
<td>77.89 (20.12)</td>
</tr>
</tbody>
</table>

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generation of DOC product ions were studied using various collision energies. No prominent product-ions were observed which is in agreement with published reports [19,20]. Based on this, MS/MS transition without fragmentation approach was followed for quantitative analysis [12,21]. Pseudo-multiple reactions monitoring mode pairs at m/z 391.24 > 391.24 was selected for monitoring DOC in the representative pneumococcal polysaccharide samples.

The optimized chromatographic conditions for RI method were initially adapted for use in MS method. The method resulted in run time of 15 min. In order to further reduce the run time, shorter length column with gradient elution using acidified water-

Fig. 2. Interference from sample matrix and sample clean up procedures. Representative HPLC-PDA chromatogram overlays to compare spin filtration and liquid–liquid extraction sample clean up procedures monitored at wavelength of 280 nm. Sample A: standard solution of DOC (2 μg/mL in methanol). Sample B: UV profile of intermediate sample of serotype 23F subjected to spin filtration, suggesting presence of interfering peak at 9.76 min. The window (W) in the figure indicates the spectra at wavelength (λ) of 201.6, 220 and 276 nm of interfering peak in sample B. Sample C: Intermediate sample spiked with DOC at 2 μg/mL and subjected to spin filtration showing the presence of interfering peak in spiked sample. Sample D and E: represents the samples B and C: subjected to liquid–liquid extraction sample clean up methodology. No interfering peaks were observed in samples processed using liquid–liquid extractions.

Fig. 3. ESI-MS of DOC in positive and negative ionization modes. Fig. A depicts the m/z 391.4 [M+H]+ in negative ionization mode. Fig B: depicts the ions at m/z 415.3 [M+Na]+; due to formation of sodium adduct of DOC (B).
methanol was explored. Various mobile phase compositions (water-methanol) with acetic acid or formic acid were studied to further improve the method sensitivity. Gradient elution using water-methanol composition with 0.1% formic acid resulted in improved peak shape, reduced run time (8 min) and sensitivity at 10 ng/mL (Fig. 4), which is in agreement with previous reports on MS analysis of free deoxycholic acid in biological matrices [11,19].

3.4. Method validation

The optimized HPLC-RI and HPLC-MS/MS methods were validated for parameters such as specificity, linearity, LOD, LOQ, accuracy and precision. The representative HPLC-RI chromatograms showed the presence of DOC at tR of 9.76 min (Fig. 1). No significant interferences from blank methanol were observed at the tR of DOC, suggesting specificity of the method. HPLC-MS/MS method did not show any interference from the product matrix, wherein DOC (10 ng/mL) peak was observed at tR of 3.66 min (Fig. 4).

The calibration curve data was fitted using linear regression analysis (Fig. 5). For HPLC-RI method, the linearity was observed over the concentration range of 2–80 μg/mL with r² = 0.999 (Table 2). Whereas, HPLC-MS/MS-based method showed linearity over the concentration range of 10–320 ng/mL with r² = 0.997. The LOD, and LOQ of 0.5 μg/mL, 2 μg/mL were observed for HPLC-RI method respectively. For HPLC-MS/MS method, the LOD and LOQ values of 3, and 10 ng/mL were obtained respectively. HPLC-MS/MS-based method was found 200 times more sensitive than the HPLC-RI method (Table 2).

Accuracy of both the methods was studied after spiking of known amount of DOC at three different concentrations (LOQ, MQC and HQC) covering the entire calibration curve range. HPLC-RI method showed accuracies in the range of 96.34–105.15%; 82.33–89.95% and 91.32–103.77% at LQC, MQC, and HQC level respectively. The overall % RSD was in the range of 0.79–5.39 suggesting the consistency of recoveries after spiking of known amount of DOC. HPLC-MS/MS-based method, recoveries were found in the range of 108.58–117.86%; 105.89–112.09% and 95.05–104.02% at LQC, MQC and HQC levels respectively. % RSD of mean recoveries at all the three levels was found in the range of 1.25–7.85 suggesting the consistency in determination of percent recoveries in spiked samples (Table 2).

For repeatability studies using HPLC-RI method, % RSD for DOC content was observed in the range of 2.49–3.24; 1.14–4.33 and 0.74–1.45 for LQC, MQC, and HQC level respectively. Whereas, intermediate precision showed % RSD in the range of 2.42–2.96; 1.93–3.95 and 0.93–1.86 for LQC, MQC, and HQC level respectively. Similarly, HPLC-MS/MS method displayed % RSDs of 1.95–8.29; 4.03–5.97 and 3.16–3.86 for LQC, MQC, and HQC level respectively.

![Fig. 4. Representative chromatograms of HPLC-MS/MS method. Figure depicts the method ability to detect the presence of DOC in crude and purified samples of serotype 9V. HPLC-ESI-MS/MS chromatograms of A) Blank; B) reference standard of DOC (at LOQ level = 10 ng/mL); C) serotype 9V crude stage sample showing the presence of DOC; D) serotype 9V purified sample representing matrix of purified sample and E) serotype 9V purified sample spiked with DOC; 20 ng/mL.](image-url)
during the repeatability study. In intermediate precision, HPLC-MS/MS method showed % RSD in the range of 3.80–7.21; 3.73–7.74 and 3.45–4.61 for LQC, MQC, and HQC level respectively (Table 2). Method validation results suggest that both methods have required specificity, sensitivity, linearity, precision and accuracy in the intended matrices.

3.5. Uncertainty of HPLC-RI and HPLC-MS/MS method

Partial and expanded uncertainties for both methods were calculated for the four different sample matrices represented by serotypes 1, 5, 9V and 23F (Table 3). Three sources of uncertainty: a) calibration curve b) precision and c) accuracy were considered for determination of expanded uncertainty. The precision and accuracy estimates were derived from LQC samples, considering as worst case scenarios. The results suggest that accuracy parameter as the major contributor to source to overall uncertainty. This is further in agreement with validation study observations where the percent recoveries in spiked samples for both methods were found in the range of 80–120%. Application of method: Determination of DOC in selected Pneumococcal polysaccharide samples. Pneumococcal vaccines are multivalent in nature and comprise of capsular polysaccharides from multiple serotypes. The validated HPLC-RI and HPLC-MS/MS methods were applied for the analysis of DOC in process intermediate and final samples of pneumococcal polysaccharides derived from representative serotypes of 1, 5, 9V, and 23F (Table 4). Both methods showed spike recoveries of 92–108% in process intermediate samples of serotypes, 1, 5, 9V and

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**Fig. 5.** Calibration curves for DOC using HPLC-RI (A) and HPLC-MS/MS method (B). For HPLC-RI method, the linearity was observed over the concentration range of 2–80 μg/mL with correlation coefficient \( r^2 = 0.999 \). Whereas, HPLC-MS/MS method showed linearity over the concentration range of 10–320 ng/mL with \( r^2 = 0.997 \).
23F suggesting its applicability for monitoring the levels of DOC during the purification. The method applicability to other pneumococcal serotypes polysaccharides was also studied. Both methods showed acceptable spike recoveries (80–120%) at LOQ levels for serotypes 6A, 6B, 7F, 14, 19A and 19F samples suggesting the universal suitability of the method for detection and quantification of DOC residues in various pneumococcal polysaccharides (Table 5). The method was also applied for on-line monitoring of DOC during the process. The HPLC-MS/MS method was able to detect traces of DOC in the initial in-process samples of serotypes 5 and 9V during the vaccine manufacturing process. Serotype 5 and 9V showed DOC content of 23.14 ± 2.89 and 109.54 ± 20.28 ng/mL respectively. These levels were further reduced to zero or below detection limits in subsequent stages of purification. No DOC was detected in final purified samples of 5 and 9V indicating the complete removal of DOC during the process. As the residual concentration of DOC during in-process and final samples are in very minute quantities (in ng/mL level), expectedly HPLC-RI method with LOQ of 2 µg/mL was not able to detect DOC at these levels.

4. Discussion and conclusion

DOC plays specific role in cell lysis and isolation of saccharides in the manufacturing of polysaccharide vaccines. Accordingly, the development of specific and sensitive analytical techniques for monitoring of residual DOC is needed. Two new, sensitive and rapid methods for quantification of DOC in vaccine matrices based on HPLC-RI and HPLC-MS/MS were developed and validated. HPLC-UV based method is reported in the literature, however, was not preferred for the intended applications as intermediate samples (deoxycholate lyases) in polysaccharide vaccines are rich in UV sensitive impurities (cellular components like membrane proteins and nucleic acids).

For MS analysis, selection of characteristic ion pairs (mostly molecular ion and their daughter ions with higher intensities) is important for the sensitivity and selectivity of the MS/MS based method. Various conditions were studied to generate the daughter ions of DOC. However, poor intensity daughter ions were observed, which was consistent with literature reports. Therefore, pseudo-multiple reactions monitoring mode (MRM) pair at m/z 391.24 > 391.24 was selected for monitoring DOC in the representative pneumococcal polysaccharide samples. . HPLC-MS based methods are undoubtedly the best analytical techniques for trace analysis, however, are capital intensive. HPLC-RI method was developed as an alternative approach with sensitivity at 2 µg/mL. The proposed RI method has considerable advantages in terms of sensitivity, specificity and run time as compared to published methods. Significant challenges of interferences from sample matrices were observed especially in intermediate stage samples. For MS analysis, selection of characteristic ion pairs (mostly molecular ion and their daughter ions with higher intensities) is important for the sensitivity and selectivity of the MS/MS based method. Two new, sensitive and rapid methods for quantification of DOC in vaccine matrices based on HPLC-RI and HPLC-MS/MS were developed and validated. HPLC-UV based method is reported in the literature, however, was not preferred for the intended applications as intermediate samples (deoxycholate lyases) in polysaccharide vaccines are rich in UV sensitive impurities (cellular components like membrane proteins and nucleic acids).

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cost effective, which is a considerable improvement over published methods. Both methods were studied for suitability with respect to ten different complex sample matrices represented by pneumococcal polysaccharides derived from serotypes 1, 5, 6A, 6B, 7F, 9V, 14, 19A, 19F and 23F. These methods were found to be specific and accurate for all the studied sample matrices. HPLC-MS/MS method was able to detect the traceable quantities of DOC (up to 10 ng/mL); suggesting its superiority for such applications. In conclusion, analytical methods reported here allow rapid (run time below 15 min) and reproducible analysis of DOC in most complex matrices of polysaccharide vaccines and thus can be readily adapted to the analysis of DOC in other vaccine matrices.

Conflict of interest statement

The authors (Sunil Gairola, Manish Gautam, Dada Patil, Krishna Manoj Kumar, S.K. Jana, Rajeev Dhere, and Suresh Jadhav) are employees of Serum Institute of India Pvt. Ltd. Pune, India. Mr. Pravin Shinde is an employee of Doctors Analytical Laboratory, Mumbai, India. All the authors declare no conflict of interest.

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