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Address of Editorial Board

Faculty of Pharmacy
2, Dunav str., Sofia 1000
Fax (02) 987 987 4

Editor in Chief: ☏(+359 2) 9236 505
E-mail: pharmacia_editor@pharmfac.net
Secretary: ☏(02) 9236 515
E-mail: pharmacia_secretary@pharmfac.net
QUALITY CONTROL OF BOUND PROTEIN IN CONJUGATED VACCINES BY SPECTROPHOTOMETRY

D. Obreshkova¹, D. Tsvetkova¹, L. Saso²

¹Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University – Sofia
²Department of Physiology and Pharmacology, Sapienza University - Rome

Abstract. The aim of current study is the determination of content of bound protein in conjugated vaccines by applying UV – spectrophotometry. The obtained regression equation: \( y = 8623.2x + 0.0507 \), shows the proportional accordance \( A = f (C) \) in linear concentration range: \( 1.10^{-5} \mu g/ml \div 9.10^{-5} \mu g/ml \). Accuracy is presented by the degree recovery \( R(\%) \pm RSD(\%) \), which for results suit relevant confidence interval: 94.09 % ÷ 102.91 % (\( C_{P17} \)); 98.91 % ÷ 103.1 % (\( C_{P40.5} \)); 99.14 % ÷ 100.30 % (\( C_{P64} \)). Precision (repeatability) is determined by: SD, RSD and confidential interval. All results for the obtained content of protein in model mixtures correspond to the relevant confidence interval: 15.13 ÷ 17.65 (\( C_{P17} \)); 39.54 ÷ 42.28 (\( C_{P40.5} \)); 63.12 ÷ 64.52 (\( C_{P64} \)). The obtained quantity protein in vaccine is \( (29.23 \mu g ÷ 36.51 \mu g)/0.5 \text{ ml} \) and suit to the higher labeled content: \( (27.2 \mu g ÷ 36.8 \mu g)/0.5 \text{ ml} \).

Key words: bound protein, conjugated vaccines, spectrophotometry.

Introduction

Streptococcus pneumoniae is the leading cause of invasive pneumococcal diseases (septicemia, meningitis and bacteraemic pneumonia) and non invasive pneumococcal diseases (acute otitis media, non-bacteraemic pneumonia, sinusitis and bronchitis) in young children and affects people with chronic obstructive pulmonary disease [1]. Polysaccharide vaccines contain purified capsular polysaccharides and induce a B-cell dependent immune response via release of immunoglobulin M (IgM). Until recently the only pneumococcal vaccine approved for use in adults in the USA and Europe is 23-valent pneumococcal polysaccharide vaccine (PPSV23) [1, 2]. Antibodies against PPSV23 antigens persist ten years after vaccination [3]. PPSV23 provides partial protection against invasive pneumococcal disease in young healthy patients and protect against pneumococcal infection in patients with lung disease [4], but appears to have limited impact on this risk among older patients. Revaccination is needed for elevation of antibody responses in adults aged 65 [5]. Unconjugated vaccines do not induce immune memory and children below 2 years of age. This limitation is overcome by covalent linkage of polysaccharide antigens to different carrier proteins: protein D from E. coli strain B1084 [6]; tetanus toxoid from C. tetani strain No 49205 Y-IV-4 or diphtheria toxoid from Corynebacterium diphtheriae strain C7 (β197). In comparison with unconjugated pneumococcal polysaccharide vaccines the developed pneumococcal protein conjugated vaccines are capable of inducing T-cell memory and are more effective against bacterial pathogens Haemophilus influenzae type b, Streptococcus pneumoniae and Neisseria meningitides, due to increased immunogenicity as a result of a link of the polysaccharide antigen to a nontoxic protein carrier [7].

Synflorix is the very often applied second generation 10-valent conjugated vaccine, composed of 10 polysaccharide serotypes, individually conjugated respectively to protein D from E. coli strain B1084 (1, 5, 6B, 7F, 9V, 14 and 23F), tetanus toxoid (18C) and diphtheria toxoid (19F). In comparison with 7-valent conjugated vaccines, Synflorix contains three more pneumococcal serotypes (1, 5 and 7F), which cause 13% of all invasive pneumococcal diseases in children below 5 years of age [8].

Other applied diphtheria protein conjugated pneumococcal vaccines are: 5-valent Prevenar 5 (PCV5): 5 serotypes (6B, 14, 18C, 19F, 23F) and 7-valent Prevenar 7: 7 serotypes (4, 6B, 9V, 14, 18C, 19F, 23F) [9]. 3) Prevenar 13: 13 serotypes: (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) [10].

Synflorix induces the more potent immune response in children than PCV7 [8, 11] and randomized
trials revealed that since the introduction of Synflorix in the USA in 2000 in children population the efficacy is 97.4% in prevention of invasive pneumococcal disease [8, 12] and 57% against vaccine serotype pneumococcal acute otitis media [8, 13]. In comparison with PCV7, Synflorix is more effective against pneumonia [8, 14] and pneumococcal meningitis [8, 15], HIV-infected patients [20, 16] and induces a greater functional antibody response than PPSV23 in adults aged 50-80 years [8, 17]. In comparison to Prevnar [7] the new serotypes added to Sinflorix are responsible for approximately 50% to 65% of the current cases of invasive pneumococcal diseases, occurring among children [8].

Free pneumococcal polysaccharides are determined by: 1) high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in polysaccharide-diphtheria toxoid conjugate vaccines [18] and in tetanus toxoid-conjugate vaccine [19, 20]; 2) HPLC with reverse phase and fluorescence detection [21]; 3) nephelometric method in a polyvalent pneumococcal conjugate vaccine with ([β197]) [22]. For the quantification of the capsular polysaccharide polyribosyl ribitol phosphate are applied: 1) HPAEC-PAD [23]; 2) phosphorus assay by inductively coupled plasma-atomic emission spectrometry haemophilus influenza type b conjugate vaccines [24].

In aluminum hydroxide (Alhydrogel) based vaccines proteins are determined by fluorescent assay using the o-phthalaldehyde reagent [25]. In protein-polysaccharide conjugate consisting of Streptococcus pneumoniae or Neisseria meningitidis polysaccharide, covalently linked to diphtheria toxoid, the free carrier protein is analysed by micellar electrokinetic chromatography with an internal standard (myoglobin), using capillary 67 cm, 350 mm i.d.; separation buffer: 2.5 ml 0.2 M sodium dodecyl sulfate : 15 ml 0.05 M sodium borate borate, pH = 9.2; voltage: 30 kV; temperature – 208°C; UV detection at λ = 200 nm [26]. In meningococcal polysaccharide-diphtheria toxoid conjugate vaccines free protein diphtheria toxoid is determined by capillary electrophoresis using alkaline (pH 9-10) borate or glycine/NaOH buffers [27].

The aim of current study is the determination of content of bound protein in conjugated Synflorix vaccines by applying UV – spectrophotometry.

Materials and methods


Method: UV-spectrophotometry.

I) Preparation of solutions of reference standard bovine serum albumine for linearity.

An accurately weighed quantity of reference standard bovine serum albumine: 1 mg, 2 mg, 3 mg, 4 mg, 5 mg, 6 mg, 7 mg, 8 mg, 9 mg was dissolved in volumetric flask to 100.0 ml with distilled water, to obtain concentrations: 1.10^-3 g/ml, 2.10^-3 g/ml, 3.10^-3 g/ml, 4.10^-3 g/ml, 5.10^-3 g/ml, 6.10^-3 g/ml, 7.10^-3 g/ml, 8.10^-3 g/ml, 9.10^-3 g/ml.

II) Preparation of model mixtures from reference standard bovine serum albumine for accuracy and precision (repeatability).

Three types equal homogenous model mixtures were prepared by dissolving in volumetric flasks of 10.0 ml in destilled water the reference standard bovine serum albumin to obtain solutions, containing the lower (P17, 17 µg), middle (P 41, 41 µg) and upper (P 64, 64 µg) labeled content of protein in conjugated vaccine.

III) Preparation of sample of conjugated vaccine.

The analysed vaccine was centrifugated for 15 min. at 5000 rpm. The obtained precipitate was resuspended in 5 ml fresh prepared desorption mixture, containing 1 ml 56 g/l solution of sodium edetate and 49 ml 90 g/l solution of disodium hydrogen phosphate. In process of desorption during incubation at 37°C for 48 h., proteins are desorbed from aluminium hydroxide. The sample was centrifugated for 15 min. at 5000 rpm.

The absorbance of all solutions was measured at λ = 280 nm, using distilled water as blank solution.

Results and discussion

In accordance to ICH guideline the validation of spectrophotometric method for analysis of protein in conjugated vaccines is presented by estimation of analytical parameters: linearity, accuracy and precision.

I) Validation of analytical parameter linearity: application of method of linear regression analysis.

For the validation of analytical parameter linearity the solutions with decreasing concentration of reference standard bovine serum albumine were analyzed...
by the written UV-spectrophotometric method. For every concentration (C) in µg/ml was measured the respective value of the absorption (A) in absorption units (AU) at λ = 280 nm. The spectra and the data for absorbances of standard solutions for linearity are illustrated on Fig. 1.

The experimental results were put into linearity regression analysis. The regression calibration curve was built. The obtained regression equation: \( y = 8623 \times + 0.0507 \), shows the proportional accordance A = f (C) in linear concentration range: 1.10–5 µg/ml ÷ 9.10–5 μg/ml, where the Buge-Lambert-Beer law is valid. Coefficient of regression (R) is calculated: \( R^2 = 0.961 \). SD for the slope is 597.498856 and SD for the intercept is 0.033623139.

The calibration curve with reference standard bovine serum albumine at λ = 280 nm is presented on Fig. 2.

II) Validation of spectrophotometric method for analytical parameters accuracy and precision (repeatability).

For model mixtures with bovine serum albumine (Fig. 3.) and for vaccine Synflorix (Fig. 4.) the spectra and absorbances λ = 280 nm are presented.

On Table 1. are summarized data for: 1) added quantity of reference standard in model mixtures: P17, P40.5, P64; 2) weighed quantity (W) of model mixtures for analysis: W P17, W P40.5, W P64; 3) values for absorbance (A) of solutions of model mixtures with in distilled water at λ = 280 nm: AP17, AP40.5, AP64; 4) Chauvenet’s criterion for absorbance (UA): UA P17, UA P40.5, UA P64.

Content of protein in model mixtures and in vaccine is obtained by method of calibration curve. On Table 2. (model mixtures) and Table 3. (vaccine) are indicated: N - number of the individual measurements (1 ÷ 3; 1 ÷ 6); C - obtained quantity of bovine serum albumine: CP17, CP40.5, CP64 and of protein in vaccine (C Synflorix); U C - Chauvenet’s criterion for C; R (%) – recovery for C; URP17, URP40.5, URP64; U C Synflorix; \( \bar{X} \) - arithmetical mean; standard (SD) and relative standard deviation (RSD) (%); S \( \bar{X} \) - mean
quadratic error; P - confidence possibility (%); t - coefficient of Student; $\bar{X} \pm t \cdot S \cdot \bar{X}$ - confidence interval (CI); E - relative error.

For all values the obtained results for Chauvenet’s criterion (Table 3.) are lower than standard UC < 1.68, which confirm, that all experimental data suit standard requirements. For the assessment of accuracy and precision is calculated sample standard deviation (SD), by the applying of the Bessel’s correction, in which the denominator N - 1 (degrees of freedom) is used instead of N and in this case (S)² is an unbiased estimator for (SD)². Analytical parameter accuracy is presented by the degree recovery R (%) ± RSD (%). Data show that at P = 92 % all results for R, suit relevant CI: 94.09 % ÷ 102.91 % (СP17); 98.91 % ÷ 103.1 % (СP40.5); 99.14 % ÷ 100.30 % (СP64). For the estimation of an analytical parameter precision (repeatability) is used the uncertainty of the result, which is determined by: SD, RSD and confidential interval. At confidence possibility P = 92 % (t = 2.92) all data for the obtained content of protein in model mixtures and conjugated vaccines correspond to the relevant CI.

Conclusion

For quality control of bound protein in conjugated vaccines is applied UV-spectrophotometric method.
Table 1. Absorbance of model mixtures of reference standard with bovine serum albumine

<table>
<thead>
<tr>
<th>N</th>
<th>$P_{17}$ [µg]</th>
<th>$W_{P_{17}}$ [g]</th>
<th>$P_{40.5}$ [µg]</th>
<th>$W_{P_{40.5}}$ [g]</th>
<th>$P_{64}$ [µg]</th>
<th>$W_{P_{64}}$ [g]</th>
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<tbody>
<tr>
<td>1</td>
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<td>0.0164</td>
<td>40.2</td>
<td>0.0402</td>
<td>63.8</td>
<td>0.0638</td>
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<tr>
<td>2</td>
<td>16.5</td>
<td>0.0165</td>
<td>40.5</td>
<td>0.0405</td>
<td>64.0</td>
<td>0.0640</td>
</tr>
<tr>
<td>3</td>
<td>17.0</td>
<td>0.017</td>
<td>40.8</td>
<td>0.0408</td>
<td>64.2</td>
<td>0.0642</td>
</tr>
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</table>

Absorbance of model mixtures of reference standard Bovine serum albumine and Chauvenet’s criterion for absorbance ($U_A$).

<table>
<thead>
<tr>
<th>N</th>
<th>$A_{P_{17}}$</th>
<th>$U_{A_{P_{17}}}$</th>
<th>$A_{P_{40.5}}$</th>
<th>$U_{A_{P_{40.5}}}$</th>
<th>$A_{P_{64}}$</th>
<th>$U_{A_{P_{64}}}$</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>0.18819</td>
<td>0.64</td>
<td>0.39639</td>
<td>1.07</td>
<td>0.59776</td>
<td>0.97</td>
</tr>
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<td>2</td>
<td>0.18898</td>
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<td>0.60112</td>
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<tr>
<td>3</td>
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<td>1.15</td>
<td>0.41014</td>
<td>0.92</td>
<td>0.60506</td>
<td>1.03</td>
</tr>
</tbody>
</table>

$\bar{X} = 0.19233$  
$SD = 0.00649$  
$RSD [%] = 3.37$  

<table>
<thead>
<tr>
<th>N</th>
<th>$C_{P_{17}}$ [µg]</th>
<th>$RC_{P_{17}}$ [%]</th>
<th>$UC_{P_{17}}$</th>
<th>$C_{P_{40.5}}$ [µg]</th>
<th>$RC_{P_{40.5}}$ [%]</th>
<th>$UC_{P_{40.5}}$</th>
<th>$C_{P_{64}}$ [µg]</th>
<th>$RC_{P_{64}}$ [%]</th>
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</thead>
<tbody>
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<td>1</td>
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<td>97.01</td>
<td>0.64</td>
<td>40.05</td>
<td>99.63</td>
<td>1.06</td>
<td>63.41</td>
<td>99.39</td>
<td>0.98</td>
</tr>
<tr>
<td>2</td>
<td>16.00</td>
<td>96.97</td>
<td>0.52</td>
<td>41.03</td>
<td>99.63</td>
<td>0.15</td>
<td>63.80</td>
<td>99.69</td>
<td>0.05</td>
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<tr>
<td>3</td>
<td>17.26</td>
<td>101.53</td>
<td>1.16</td>
<td>41.65</td>
<td>102.08</td>
<td>0.91</td>
<td>64.25</td>
<td>100.08</td>
<td>1.02</td>
</tr>
</tbody>
</table>

$\bar{X} \pm SD = 16.39 \pm 0.75$  
$RC_{P_{17}} \pm RSD [%] = 98.50 \pm 2.66$  
$UC_{P_{17}} \pm RSD [%] = 101.01 \pm 1.24$  
$RC_{P_{40.5}} \pm RSD [%] = 99.72 \pm 0.35$  

<table>
<thead>
<tr>
<th>N</th>
<th>$P_{17}$ [%]</th>
<th>$UC_{P_{17}}$</th>
<th>$P_{40.5}$ [%]</th>
<th>$UC_{P_{40.5}}$</th>
<th>$P_{64}$ [%]</th>
<th>$UC_{P_{64}}$</th>
</tr>
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<tbody>
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<td>90.0</td>
<td>92.0</td>
<td>92.0</td>
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<td>92.0</td>
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<tr>
<td>2</td>
<td>90.0</td>
<td>90.0</td>
<td>92.0</td>
<td>92.0</td>
<td>92.0</td>
<td>92.0</td>
</tr>
<tr>
<td>3</td>
<td>90.0</td>
<td>90.0</td>
<td>92.0</td>
<td>92.0</td>
<td>92.0</td>
<td>92.0</td>
</tr>
</tbody>
</table>

$t = 2.92$  
$t.S\bar{X} = 0.43$  
$t.S\bar{X} + t.S\bar{X} = 15.13 \div 17.65$  
$E [%] = 2.62$  

Table 2. Obtained quantity ($C$) of bovine serum albumine in model mixtures, recovery for $C$ ($RC$) (%) and Chauvenet’s criterion for $C$ ($UC$)

<table>
<thead>
<tr>
<th>N</th>
<th>$C_{P_{17}}$ [µg]</th>
<th>$RC_{P_{17}}$ [%]</th>
<th>$UC_{P_{17}}$</th>
<th>$C_{P_{40.5}}$ [µg]</th>
<th>$RC_{P_{40.5}}$ [%]</th>
<th>$UC_{P_{40.5}}$</th>
<th>$C_{P_{64}}$ [µg]</th>
<th>$RC_{P_{64}}$ [%]</th>
<th>$UC_{P_{64}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.91</td>
<td>97.01</td>
<td>0.64</td>
<td>40.05</td>
<td>99.63</td>
<td>1.06</td>
<td>63.41</td>
<td>99.39</td>
<td>0.98</td>
</tr>
<tr>
<td>2</td>
<td>16.00</td>
<td>96.97</td>
<td>0.52</td>
<td>41.03</td>
<td>99.63</td>
<td>0.15</td>
<td>63.80</td>
<td>99.69</td>
<td>0.05</td>
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<tr>
<td>3</td>
<td>17.26</td>
<td>101.53</td>
<td>1.16</td>
<td>41.65</td>
<td>102.08</td>
<td>0.91</td>
<td>64.25</td>
<td>100.08</td>
<td>1.02</td>
</tr>
</tbody>
</table>

$t = 2.92$  
$t.S\bar{X} = 0.43$  
$t.S\bar{X} + t.S\bar{X} = 15.13 \div 17.65$  
$E [%] = 2.62$
Quality control of bound protein in conjugated vaccines

**Table 3. Absorbance and content of protein in vaccine**

<table>
<thead>
<tr>
<th>N:</th>
<th>A_{Synflorix + Al susp.}</th>
<th>U_{Synflorix + Al susp.}</th>
<th>A_{Synflorix}</th>
<th>U_{A_{Synflorix}}</th>
<th>C_{Synflorix}</th>
</tr>
</thead>
<tbody>
<tr>
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<td>28.93</td>
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<td>0.60</td>
<td>0.32070</td>
<td>0.60</td>
<td>31.28</td>
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<tr>
<td>3.</td>
<td>0.49516</td>
<td>0.19</td>
<td>0.32994</td>
<td>0.19</td>
<td>32.35</td>
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<tr>
<td>4.</td>
<td>0.50185</td>
<td>0.10</td>
<td>0.33663</td>
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<td>33.12</td>
</tr>
<tr>
<td>5.</td>
<td>0.52422</td>
<td>1.08</td>
<td>0.35900</td>
<td>1.08</td>
<td>35.72</td>
</tr>
<tr>
<td>6.</td>
<td>0.52483</td>
<td>1.10</td>
<td>0.35961</td>
<td>1.10</td>
<td>35.79</td>
</tr>
<tr>
<td>(\bar{X})</td>
<td>0.49961</td>
<td></td>
<td></td>
<td></td>
<td>32.87</td>
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<tr>
<td>SD</td>
<td>0.02283</td>
<td></td>
<td></td>
<td></td>
<td>2.65</td>
</tr>
<tr>
<td>RSD [%]</td>
<td>4.57</td>
<td></td>
<td></td>
<td></td>
<td>8.06</td>
</tr>
</tbody>
</table>

All data for the obtained content of protein in model mixtures and in the analyzed Synflorix vaccine correspond to the relevant confidence interval: 15.13 ÷ 17.65 (C_{p_{17}}), 39.54 ÷ 42.28 (C_{p_{40.5}}), 63.12 ÷ 64.52 (C_{p_{64}}). The obtained quantity protein in Synflorix vaccine is (29.23 µg ÷ 36.51 µg)/0.5 ml and suit to the upper range of the labeled content: (27.2 µg ÷ 36.8g)/0.5 ml. The applied method for analysis of proteins in Synflorix vaccine is appropriate for quality control of bound proteins in different types conjugated vaccines.

**References**


**Notes:**

**Corresponding author**
Medical University – Sofia, Faculty of Pharmacy, Department of Pharmaceutical chemistry, Tel: +3599236530
e-mail: phddanka@yahoo.com