PHARMACIA

Volume 60 **2013** **Number 2**

JOURNAL OF THE BULGARIAN PHARMACEUTICAL SCIENTIFIC SOCIETY

Indexed in: MEDLINE, CAplus[SM]/Chemical Abstracts, TOXCENTER, EMBASE/Excerpta Medica, PASCAL, BIOTECHNOBASE, ExtraMED[TM], SCOPUS

Editorial/publishing policy: Manuscripts submitted to PHARMACIA are only accepted on the understanding, that they are subject to editorial review and review of at least two independent referees, that they have not been and will not be bublished whole or in part in any other journal and that recommendations to comply with with ethycal standards when performing clinical and other biological experiments have been adhered to.

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. Obreshkova[1], D. Tsvetkova[1], L. Saso[2]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University – Sofia
[2]Department 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.x + 0.0507, shows the proportional accordance A = f (C) in linear concentration range: 1.10^{-5} μg/ml ÷ 9.10^{-5} μg/ml. Accuracy is presented by the degree recovery R (%) ± 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 μg ÷ 36.51 μg)/0.5 ml and suit to the higher labeled content: (27.2 μg ÷ 36.8 μg)/0.5 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 (sacute 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 overcomed 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 developped 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) Prevnar 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 meningiditis 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

Materials: I) Reference standard: bovine serum albumine. II) Reagents with analytical grade quality: sodium edentate, disodium hydrogen phosphate, aluminium hydroxide, distilled water. III) Synflorix vaccine batch N: ASPNA259AE (Glaxo Smith Cline).

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 obtaine concentrations: 1.10^{-5} g/ml, 2.10^{-5} g/ml, 3.10^{-5} g/ml, 4.10^{-5} g/ml, 5.10^{-5} g/ml, 6.10^{-5} g/ml, 7.10^{-5} g/ml, 8.10^{-3} g/ml, 9.10^{-5} g/ml.

II) Preparation of model mixtures from reference standard bovine serum albumine for accurasy 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 (P_{17}, 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 desorbtion 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 desorbtion during incubation at 37°C for 48 h., proteins are desorbed from aluminium hydroxide. The sample was centrifugated for 15 min. at 5000 rpm.

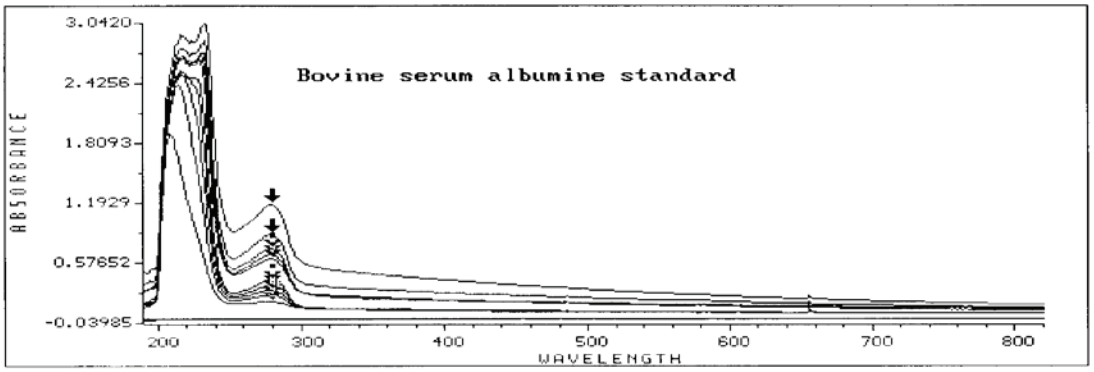
The absorbance of all solutions was measured at λ = 280 nm, using distilled water as blank solution.

Results and discussion

In accordansce 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



Marked Wavelengths

Reg K:	L 280 =	1.1720
Reg M:	L 280 =	0.86617
Reg O:	L 280 =	0.72108
Reg Q:	L 280 =	0.66075
Reg S:	L 280 =	0.61739
Reg A:	L 280 =	0.40503
Reg C:	L 280 =	0.34923
Reg E:	L 280 =	0.29445
Reg G:	L 280 =	0.24574
Reg I:	L 280 =	0.17941

Fig.1. *Spectra for absorbances of reference standard bovine serum albumine*

by the written UV- spectrophotometric method. For every concentration (C) in μg/ml was measured the respective value of the absorbtion (A) in absorbtion 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 putted into linearity regression analysis. The regression calibration curve was built. The obtained regression equation: y = 8623.x + 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 - Beere 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.

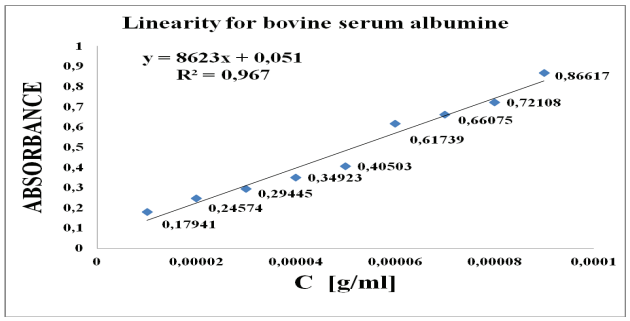


Fig.2. *Calibration curve for linearity for bovine serum ablumine at λ = 280 nm.*

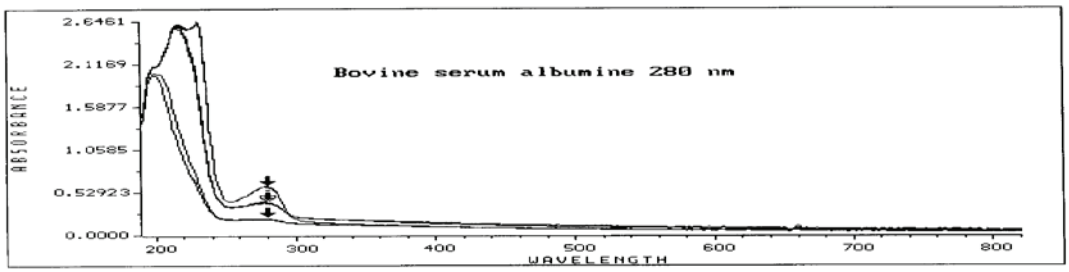
The calibration curve with reference standard bovine serum ablumine at λ = 280 nm is presented on Fig. 2.

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.

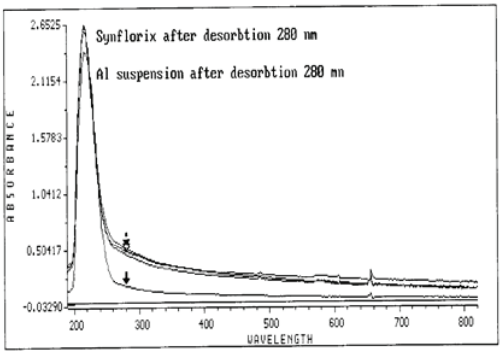
On Table 1. are summarized data for: 1) added quantity of reference standard in model mixtures: $P_{17,}$ $P_{40.5,}$ P_{64}; 2) weighed quantity (W) of model mixtures for analysis: W $P_{17,}$ W $P_{40,5,}$ W P_{64}; 3) values for absorbance (A) of solutions of model mixtures with in distilled water at λ = 280 nm: $A_{P17,}$ $A_{P40.5,}$ A_{P64}; 4) Chauvenet′s criterion for absorbance (UA): U $A_{P17,}$ U $A_{P40.5,}$ U $A_{P64.}$

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: $CP_{17,}$ $CP_{40.5,}$ CP_{64} and of protein in vaccine ($C_{Synflorix}$); UC - Chauvenet′s criterion for C: R (%) – recovery for C: $URP_{17,}$ $URP_{40.5,}$ URP_{64}; U $C_{Synflorix}$; $\overline{X}$ - arithmetical mean; standard (SD) and relative standard deviation (RSD) (%); S $\overline{X}$ - mean

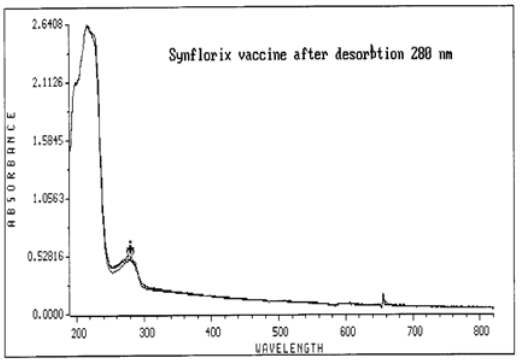


Marked Wavelengths
Reg S: L 280 = 0.60506
Reg P: L 280 = 0.60112
Reg R: L 280 = 0.59776
Reg B: L 280 = 0.40483
Reg T: L 280 = 0.41014
Reg C: L 280 = 0.39639
Reg M: L 280 = 0.19981
Reg N: L 280 = 0.18898
Reg O: L 280 = 0.18819

Fig.3. *Spectra and absorbances of model mixtures of bovine serum albumine*



Marked Wavelengths
Reg D: L 280 = 0.16522
Reg K: L 280 = 0.52483
Reg H: L 280 = 0.49516
Reg J: L 280 = 0.46568



Marked Wavelengths
Reg G: L 280 = 0.52422
Reg H: L 280 = 0.50185
Reg I: L 280 = 0.48592

Fig.4. *Spectra and absorbances for vaccine*

quadratic error; P - confidence possibility (%); t - coefficient of Student; $\overline{X} \div t.S\overline{X}$ - confidence interval (CI); E - relative error.

For all values the obtained results for Chauvenet's criterion (Table 3.) are lower than tandard 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 % (C_{P17}); 98.91 % ÷ 103.1 % ($C_{P40.5}$); 99.14 % ÷ 100.30 % (C_{P64}). 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*

Added content of reference standard bovine serum albumine in model mixtures and weighed quantity of model mixtures.						
N :	P_{17} [µg]	W P_{17} [g]	$P_{40.5}$ [µg]	W $P_{40.5}$ [g]	P_{64} [µg]	W P_{64} [g]
1.	16.4	0.0164	40.2	0.0402	63.8	0.0638
2.	16.5	0.0165	40.5	0.0405	64.0	0.0640
3.	17.0	0.017	40.8	0.0408	64.2	0.0642
Absorbanse of model mixtures of reference standard Bovine serum albumine and Chauvenet′s criterion criterion for absorbanse (U A).						
N :	A_{P17}	U A_{P17}	$A_{P40.5}$	U $AP_{40.5}$	A_{P64}	U A_{P64}
1.	0.18819	0.64	0.39639	1.07	0.59776	0.97
2.	0.18898	0.52	0.40483	0.15	0.60112	0.05
3.	0.19981	1.15	0.41014	0.92	0.60506	1.03
$\overline{X}$	0.19233		0.40379		0.60131	
SD	0.00649		0.00693		0.00365	
RSD [%]	3.37		1.72		0.61	

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

N:	C_{P17} [µg]	RC_{P17} [%]	UC_{P17}	$C_{P40.5}$ [µg]	$RC_{P40.5}$ [%]	$UC_{P40.5}$	C_{P64} [µg]	RC_{P6} [%]	UC_{P64}
1.	15.91	97.01	0.64	40.05	99.63	1.06	63.41	99.39	0.98
2.	16.00	96.97	0.52	41.03	101.31	0.15	63.80	99.69	0.05
3.	17.26	101.53	1.16	41.65	102.08	0.91	64.25	100.08	1.02
$\overline{X}$ ± SD	16.39 ± 0.75			40.91 ± 0.81			63.82 ± 0.42		
$\bar{R}$ [%] ± RSD [%]		98.50 ± 2.66			101.01 ± 1.24			99.72 ± 0.35	
SD	0.75	2.62		0.81	1.25		0.42	0.35	
RSD [%]	4.58	2.66		1.98	1.24		0.66	0.55	
S $\overline{X}$	0.43	1.51		0.47	0.72		0.24	0.20	
P [%]	90.0	90.0		92.0	92.0		92.0	92.0	
t	2.92	2.92		2.92	2.92		2.92	2.92	
t.S $\overline{X}$	0.43	4.41		1.37	2.10		0.70	0.58	
$\overline{X}$ – t.S $\overline{X}$ ± $\overline{X}$ + t.S $\overline{X}$	15.13 ÷ 17.65	94.09 ÷ 102.91		39.54 ÷ 42.28	98.91 ÷ 103.11		63.12 ÷ 64.52	99.14 ÷ 100.30	
E [%]	2.62	1.53		1.15	0.71		0.38	0.2	

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

N:	$A_{Synflorix + Al susp.}$	$U_{Synflorix + Al susp.}$ $UC_{Synflorix}$	$A_{Synflorix}$	$UA_{Synflorix}$	$C_{Synflorix}$
1.	0.46568	1.49	0.30046	1.49	28.93
2.	0.48592	0.60	0.32070	0.60	31.28
3.	0.49516	0.19	0.32994	0.19	32.35
4.	0.50185	0.10	0.33663	0.10	33.12
5.	0.52422	1.08	0.35900	1.08	35.72
6.	0.52483	1.10	0.35961	1.10	35.79
$\overline{X}$	0.49961				32.87
SD	0.02283				2.65
RSD [%]	4.57				8.06

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_{P17}); 39.54 ÷ 42.28 ($C_{P40.5}$), 63.12 ÷ 64.52 (C_{P64}). 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

1. Musher DM, Sampath R, Rodriguez-Barradas MC. The potential role for protein-conjugate pneumococcal vaccine in adults: what is the supporting evidence? Clin Infect Dis 2011; 52(5): 633-640.
2. Singleton RJ, Butler JC, Bulkow LR, Hurlburt D, O'Brien KL, Doan W, Parkinson AJ, Hennessy TW. Invasive pneumococcal disease epidemiology and effectiveness of 23-valent pneumococcal polysaccharide vaccine in Alaska native adults. Vaccine 2007; 25(12): 2288-2295.
3. Musher DM, Manoff SB, McFetridge RD, Liss C, Marchese RD, Raab J, Rueda AM, Walker ML, Hoover PA. Antibody persistence ten years after first and second doses of 23-valent pneumococcal polysaccharide vaccine, and immunogenicity and safety of second and third doses in older adults. Human Vaccines 2011; 7(9): 919-928.
4. Schenkein JG, Nahm MH, Dransfield MT. Pneumococcal vaccination for patients with COPD: current practice and future directions. Chest 2008; 133(3): 767-774.
5. Manoff SB, Liss C, Caulfield MJ, Marchese RD, Silber J, Boslego J, Romero-Steiner S, Rajam G, Glass NE, Whitney CG, Carlone GM. Revaccination with a 23-valent pneumococcal polysaccharide vaccine induces elevated and persistent functional antibody responses in adults aged 65 > or = years. J Infect Dis 2010; 201(4): 525-533.
6. Foresgren A, Riesbeck K, Janson H. Protein D of Haemophilus influenzae:a protective nontypable H. influenzae antigen and a carrier for pneumococcal conjugate vaccines. Clin Infect Dis 2008; 46(5): 726-731.
7. Poehling KA, Talbot TR, Griffin MR, Craig AS, Whitney CG, Zell E, Lexau CA, Thomas AR, Harrison LH, Reingold AL, Hadler JL, Farley MM, Anderson BJ, Schaffner W. Invasive pneumococcal disease among infants before and after introduction of pneumococcal conjugate vaccine. JAMA 2006; 295(4): 1668-1674.
8. Vesikari T, Wysocki J, Chevallier B, Karvonen A, Czajka H, Arsène JP, Lommel P, Dieussaert I, Schuerman L. Immunogenicity of the 10-valent pneumococcal non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV) compared to the licensed 7 vCRM vaccine. Pediatr Infect Dis J 2009; 28(4 Suppl): S66-S76.

9. O'Brien KL, Moulton LH, Reid R, Weatherholtz R, Oski J, Brown L, Kumar G, Parkinson A, Hu D, Hackell J, Chang I, Kohberger R, Siber G, Santosham M. Efficacy and safety of seven-valent conjugate pneumococcal vaccine in American Indian children: group randomised trial. Lancet 2003; 362(9381): 355-361.
10. Fernsten P, Mason KW, Yu X, Tummolo D, Belanger KA, Tsao H, Zhu D, Cooper D, Hagen M, Jansen KU. 13-valent pneumococcal conjugate vaccine immune sera protects against pneumococcal serotype 1, 3, and 5 bacteremia in a neonatal rat challenge model. Hum Vaccin. 2011; (Suppl 7): 75-84.
11. Black S, Shinefield H, Fireman B, Lewis E, Ray P, Hansen JR, Elvin L, Ensor KM, Hackell J, Siber G, Malinoski F, Madore D, Chang I, Kohberger R, Watson W, Austrian R, Edwards K. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. The Pediatric Infectious Disease Journal 2000; 19(3): 187-195.
12. Whitney CG, Pilishvili T, Farley MM, Schaffner W, Craig AS, Lynfield R, Nyquist AC, Gershman KA, Vazquez M, Bennett NM, Reingold A, Thomas A, Glode MP, Zell ER, Jorgensen JH, Beall B, Schuchat A. Effectiveness of seven-valent pneumococcal conjugate vaccine against invasive pneumococcal disease: a matched case-control study. Lancet. 2006; 368(9546): 1495-1502.
13. McEllistrem MC, Adams J, Mason EO, Wald ER. Epidemiology of acute otitis media caused by Streptococcus pneumoniae before and after licensure of the 7-valent pneumococcal protein conjugate vaccine. J Infect Dis 2003; 188(11): 1679-1684.
14. Hansen J, Steven S, Shinefield H, Cherian T, Benson J, Fireman B, Lewis E, Ray P, and Lee, J. Effectiveness of Heptavalent Pneumococcal Conjugate Vaccine in Children Younger Than 5 Years of Age for Prevention of Pneumonia.Updated Analysis Using World Health Organization StandardizedInterpretation of Chest Radiographs. Pediatr Infect Dis J 2006; 25(9): 779-781.
15. Hsu HE, Shutt KA, Moore MR, Beall BW, Bennett NM, Craig AS, Farley MM, Jorgensen JH, Lexau CA, Petit S, Reingold A, Schaffner W, Thomas A, Whitney CG, Harrison LH. Effect of pneumococcal conjugate vaccine on pneumococcal meningitis. N Engl J Med 2009; 360(3): 244-256.
16. Lu C-L, Hung C-C, Chuang Y-C, Liu W-C, Su C-T, Su Y-C, Chang S-F, Chang S-Y, Chang S-C. Serologic response to primary vaccination with 7-valent pneumococcal conjugate vaccine is better than with 23-valent pneumococcal polysaccharide vaccine in HIV-infected patients in the era of combination antiretroviral therapy Human Vaccines Immunotherapeutics 2013; 9(2): 398-404.
17. Goldblatt D, Southern J, Andrews N, Ashton L, Burbidge P, Woodgate S, Pebody R, Miller E. The immunogenicity of 7-valent pneumococcal conjugate vaccine versus 23-valent polysaccharide vaccine in adults aged 50–80 years. Clin Infect Dis 2009; 49(9): 1318-1325.
18. Lei QP, Shannon AG, Heller RK, Lamb DH.Quantification of free polysaccharide in meningococcal polysaccharide-diphtheria toxoid conjugate vaccines. Dev Biol (Basel) 2000; 103: 259-264.
19. Talaga P, Vialle S, Moreau M. Development of a high-performance anion-exchange chromatography with pulsed-amperometric detection based quantification assay for pneumococcal polysaccharides and conjugates. Vaccine 2002; 20(19-20): 2474-2484.
20. Lei QP, Lamb DH, Heller R, Pietrobon P. Quantitation of low level unconjugated polysaccharide in tetanus toxoid-conjugate vaccine by HPAEC/PAD following rapid separation by deoxycholate/HCl. J Pharm Biomed Anal 2000; 21(6): 1087-1091.
21. Canaán-Haden L, Cremata J, Chang J, Valdés Y, Cardoso F, Bencomo VV. High-performance reverse phase chromatography with fluorescence detection assay for characterization and quantification of pneumococcal polysaccharides. Vaccine 2006; 24 (Suppl 2): S2-70-71.
22. Lee CJ. Quality control of polyvalent pneumococcal polysaccharide-protein conjugate vaccine by nephelometry. Biologicals 2002; 30(2): 97-103.
23. Belfast M, Lu R, Capen R, Zhong J, Nguyen MA, Gimenez J, Sitrin R, Mancinelli R.. A practical approach to optimization and validation of a HPLC assay for analysis of polyribosyl-ribitol phosphate in complex combination vaccines. J Chromatogr B Analyt Technol Biomed Life Sci 2006; 832(2): 208-215.

24. Swartz LA, Progar JJ, May JC. The determination of phosphorus in haemophilus influenza type b conjugate vaccines by inductively coupled plasma-atomic emission spectrometry. Biologicals 2000, 28(4), 227-231.
25. Zhu D, Saul A, Huang S, Martin LB, Miller LH, Rausch KM. Use of o-phthalaldehyde assay to determine protein contents of Alhydrogel-based vaccines. Vaccine 2009; 27(43): 6054-6059.
26. Lamb DH, Summa L, Lei QP, Duval G, Adam O. Determination of free carrier protein in protein-polysaccharide conjugate vaccines by micellar electrokinetic chromatography. J Chromatogr A 2000; 894(1-2): 311-318.
27. Lamb DH, Summa L, Lei QP. Capillary electrophoretic analysis of meningococcal polysaccharide-diphtheria toxoid conjugate vaccines. Dev Biol (Basel) 2000; 103: 251-258.

Notes:

The manuscript is in connection with Twinning Contract "Quality Control Tests for Human Vaccines and Sera" Eur – TR/09/IB/FI/01/R, 2012/2013.

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