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Research paper

Development of a new, simple, rapid ultra-high-performance liquid chromatography (UHPLC) method for the quantification of 2-phenoxyethanol in vaccines for human use

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ABSTRACT

A new, simple and rapid method for the quantitative determination of the antimicrobial preservative 2-phenoxyethanol, based on reverse phase ultra-high-performance liquid chromatography has been developed. The validation was performed according the ICH Q2 guideline "Validation of Analytical Procedures". The desired chromatographic separation was achieved on a Waters Symmetry C18 (150 × 4.6 mm, 5 μm) column using an isocratic elution, with detection at 270 nm wavelength. The mobile phase consisted of acetonitrile/water (55:45, v/v), pumped at a flow rate of 1 mL/min. The calibration curve and the analytical procedure are linear (r^2 = 0.999) from the concentration of 0.07 mg/mL to 1.1 mg/mL. The percent relative standard deviation for intra- and inter-day precision was <1%. The recovery of 2-phenoxyethanol in vaccines ranged between 96.5 and 100.60%. The limits of detection and quantitation were 1.3×10^{-4} and 2.7×10^{-4} mg/mL, respectively. The method was found to be robust by changing the column working temperature, the percentage of acetonitrile of the mobile phase and the flow rate. The validated method can be successfully and reliably used to quantify as well as to exclude presence of 2-phenoxyethanol preservative in marketed vaccines.

1. Introduction

Phenoxyethanol is widely used as a preservative in vaccines. At present more than 20 thousand cosmetic and pharmaceutical preparations contain this preservative [1], due to its broad antimicrobial spectrum along with good stability and non-volatility [2]. Antimicrobial preservatives as 2-PE are used also to prevent spoilage or adverse effects caused by microbial contamination occurring during the use of vaccines manufactured in multi-dose vials presentation. Numerous methods based on gas chromatography, HPLC and HPLC-MS/MS have been reported for the determination of 2-PE in a wide variety of matrices [3–5]. Determination of 2 PE with solid phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS/MS) detection has also been reported [2], but this technique is not commonly used in pharmaceutical quality control laboratories.

To our knowledge, no UHPLC method has been so far developed for the determination of 2-phenoxyethanol in human vaccines. Bipin Sharma et al. reported an HPLC method to determine 2-PE in a combined diphtheria, tetanus, whole pertussis vaccine [6]. Considering the wide range of antigens, adjuvants and excipients contained in marketed vaccines, the scope of the present study was to develop and validate a new method by using the reversed-phase ultra high-performance liquid chromatographic (RP-UHPLC) for routine quality control analysis of 2-PE in vaccines for human use.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN) for HPLC was from VWR Chemicals BRD (Fontenay-sous-Bois, FR).

2 phenoxyethanol (2 PE) standard was obtained from the European Pharmacopoeia, Council of Europe, with a purity ≥ 99.5%, and a density of 1.1094 g/cm^3 (1109.4 mg/mL).

Ultra-pure water (MilliQ water) was purified in our laboratory by a Millipore system (model Milli-Q Integral 3, Millipore, France).

Abbreviations: ACN, acetonitrile; 2-PE, phenoxyethanol; RT, retention time.

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Table 1
Vaccine active ingredients, excipients and their use in the method validation experiments.

Vaccine active ingredients	Adjuvant	Excipients	Use in the experiment for
Diphtheria and tetanus purified toxoids, Pertussis toxoid and Haemoagglutinin, Inactivated Polio Virus antigen D type 1,2,3 (DTaP-IPV)	Aluminum hydroxide	**2-phenoxyethanol** (2.0–3.0 μL/dose), formaldehyde, ethanol, medium 199 in water for injections. Medium 199 is a complex mixture of amino acids (including phenylalanine), mineral salts, vitamins and other substances (such as glucose) diluted in water for injections	Precision (repeatability and intermediate precision) and identity
Inactivated Polio Virus antigen D type 1,2,3 (IPV)		**2-phenoxyethanol** (2.0–3.0 μL/dose), formaldehyde, medium 199 (contains sodium and potassium).	Precision (repeatability and intermediate precision) and identity
Inactivated Hepatitis A virus (HepA)	Aluminum hydroxide	Sodium dihydrogen phosphate, sodium chloride and water	Accuracy, LOD, LOQ, Linearity
Influenza vaccine - surface type A and B antigens, inactivated (FLU)		Sodium chloride, potassium chloride, potassium dihydrogen phosphate, dibasic sodium phosphate dihydrate, magnesium chloride hexahydrate, calcium chloride dihydrate and water for injections	Specificity
Tetanus purified toxoids (T)	Aluminum hydroxide	Sodium chloride, water for injections; formaldehyde not exceeding 0.001 mg (residual from the production process)	Specificity
Diphtheria and Tetanus purified toxoids, Pertussis toxoid, Haemoagglutinin, Pertactin (DTaP)	Aluminum hydroxide and aluminum phosphate	Sodium chloride and water for injections	Specificity
Diphtheria and Tetanus purified toxoids (DT)	Aluminum hydroxide	Sodium chloride, water for injections; formaldehyde not exceeding 0.001 mg (residual from the production process)	Specificity
Diphtheria and Tetanus purified toxoids, Pertussis toxoid, Haemoagglutinin, Inactivated Polio Virus antigen D type 1,2,3, Hepatitis B antigen *H.influenzae* type b glycoconjugate (DTaP-IPV-HepB-Hib)	Aluminum hydroxide	Dibasic sodium phosphate, monobasic potassium phosphate, trometamol, sucrose, essential amino acids including L-phenylalanine and water for injections. The vaccine may contain traces of glutaraldehyde, formaldehyde, neomycin, streptomycin and polymyxin B.	Specificity
Pneumococcal glycoconjugates (7 valent)	Aluminum phosphate	Sodium chloride and water for injections	Specificity
H.influenzae type b glycoconjugate	Aluminum phosphate	Sodium chloride, monobasic sodium phosphate, dibasic sodium phosphate dihydrate, polysorbate 80, water for injections.	Specificity

2.2. Samples

Test samples were commercially available human vaccines without and with 2 – PE. The detailed composition is reported in Table 1. Vaccines without 2-PE were used to assess the matrix (excipients) influence on the quantification of 2-PE.

2.3. Preparation of 2-PE and samples solutions

A 2-PE standard stock solution of 11 mg/mL was prepared by diluting the standard in MilliQ water and aliquoted in vials, stored at +4 °C up to 1 year.

A calibration curve was built using five concentrations of 2- PE in MilliQ Water: 1.100 mg/mL, 0.550 mg/mL, 0.275 mg/mL, 0.137 mg/mL and 0.068 mg/mL.

Vaccine samples without adjuvant were used without any pretreatment. All vaccines used to assess the specificity of the method, were not diluted; HepA vaccine without 2-PE, used in the accuracy, LOD, LOQ and linearity studies, was spiked with 2-PE Standard solution to obtain a final concentration of 1.1 mg/mL; vaccines samples used in the precision and identity study (IPV and DTaP-IPV, both containing 2-PE), were diluted with MilliQ water (1:10). Ten mcL of non-adjuvanted vaccine samples were used as such for the analysis. Vaccine samples containing adjuvant were centrifuged at 5000 rpm for 10 min and 10 mcL of the supernatant were used for the analysis.

2.4. UHPLC instrumentations and chromatographic conditions

The chromatographic determination was performed with an Agilent 1290 Infinity UHPLC system (Agilent Technologies, Hewlett-Packard-Strasse 876337 Waldron, Germany) controlled by the ChemStation software. RP-UHPLC analysis was performed isocratically at 25 °C using a Symmetry300C18 Waters (150 × 4.6 mm, 5 μm) UHPLC column. The mobile phase was obtained by direct mixing in the instrument of acetonitrile and water (55:45, v/v). The total analysis time was 5 min per sample. The flow rate was 1 mL/min and the injection volume was 10 μL. 2-PE elution was monitored with an UV detector set at 270 nm.

2.5. Method validation

The linearity test was performed using five different amounts of 2-PE standards diluted in a single vaccine matrix (HepA). Solutions corresponding to each concentration level were injected in triplicate and linear regression (r^2) of the 2-PE mean peak area (y) versus 2-PE concentration (x) was calculated.

Recovery studies (accuracy) were performed using Hep A vaccine (without 2-PE) to reveal any possible interference by a different matrix (Table 1). The 2-PE stock solution (11 mg/mL) was diluted in vaccines without 2-PE, to 1.100 mg/mL, 0.550 mg/mL, 0.275 mg/mL, 0.137 mg/mL and 0.068 mg/mL. These solutions were injected in triplicate and the percentage of recoveries of the response factor (area/concentration) was calculated.

Table 2
Analytical parameters and relative acceptance criteria established for the validation of the method.

Analytical Parameters	Acceptance criteria
Linearity of the Analytical Procedure and of Calibration Curve	$r^2 > 0.995$
Accuracy	Recovery percentage must be between 80 and 120%
Precision - Repeatability	RSD% < 5% on the peak area RSD% < 5% on the concentrations
Intermediate Precision	RSD% < 5% on the peak area RSD% < 5% on the concentrations
Robustness	$r^2 > 0.995$
Detection Limit (LOD)	Through a visual evaluation, the 2-PE peak must be detectable in all three replicates
Quantitation Limit (LOQ)	2-PE peak must be detectable and quantifiable with precision and accuracy: RSD% <5% Recovery % 100 ± 20%
Specificity and Identity	Specificity: Absence of 2-PE peak in vaccines that do not contain it; Identity: The RT Ratio of 2-PE peak between Ph. Eur. Standard and vaccine 2-PE containing, must be between 0.90 and 1.10.

The precision of the method was determined as repeatability (intra-day variation) and intermediate precision (inter-day variation). Repeatability was examined by analyzing six determinations of the same sample diluted assuming compliance to the manufacturing release specification (4.4–6.6 mg/mL), in order to dilute the vaccine at a target concentration centered in assay range. The relative standard deviation (RSD) of the peak areas and of the 2-PE concentration were calculated. The inter-day variation was studied running the repeatability scheme on three different days.

Regarding the specificity of the method, vaccines without 2-PE were used to verify the absence of interfering substances displaying peaks at the same retention time of 2-PE.

The identity was evaluated comparing the retention time of 2-PE peaks in two vaccines samples containing 2-PE and in the 2-PE Pharmacopoeia Standard, in order to verify that they corresponded.

The detection (LOD) and quantitation limits (LOQ), were determined by diluting the stock solution to 1:100 mg/mL in 2-PE free vaccine. Then, further two fold dilutions in vaccine were carried out down to the concentration of 2.09×10^{-6} mg/mL. The analysis was performed in triplicate in order to evaluate the precision and accuracy of the measurement.

The robustness of method was evaluated by changing the flow rate, the mobile phase percentage of ACN and the column temperature.

2.6. Data analysis

For each analysis the retention time (RT), the area and the height of the 2-PE peak were determined.

For each validation parameter an experimental design and predetermined acceptance criteria were defined (Table 2).

The peak area was used to determine the vaccines concentration, through interpolation with the calibration curve.

3. Results

In Fig. 1 is reported an example of the chromatographic profile of the 2-PE Pharmacopoeia Standard in water by using the Symmetry300C18 Waters column (Fig. 1A) and by injecting the vaccine sample DTaP-IPV containing 2-PE in a quite complex matrix (Fig. 1B) and the vaccine IPV (Fig. 1C). In all three cases, 2-PE eluted at 1.6 min showing that the retention time is not disturbed or affected by the matrix of the vaccine.

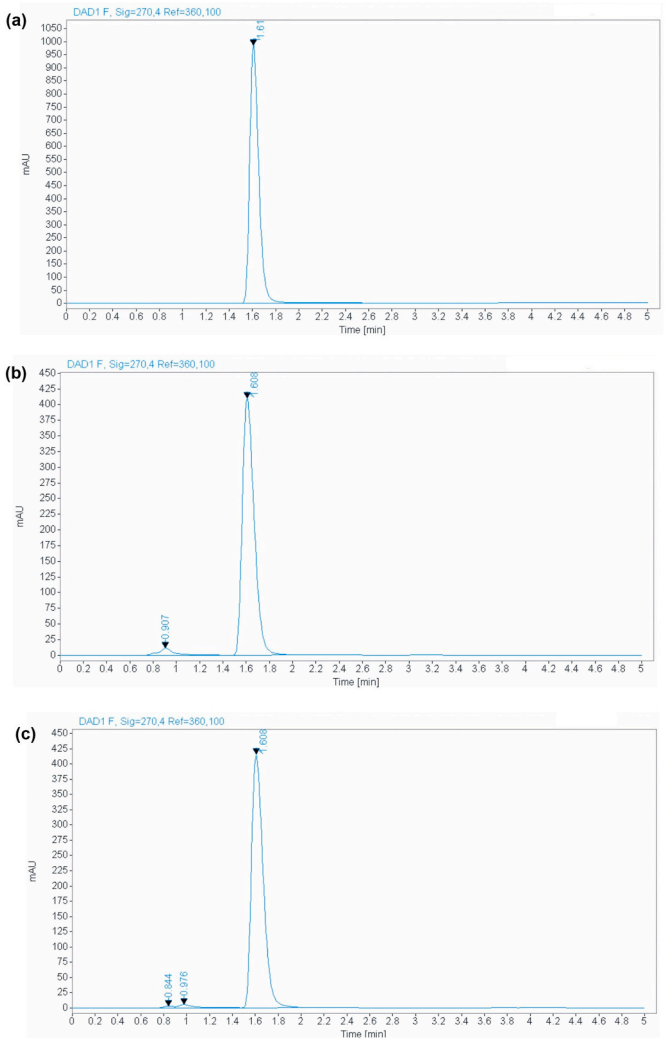

Fig. 1. 2-PE chromatographic profile in (A) 2-PE Pharmacopoeia Standard diluted in water for injection; (B) 2-PE contained in DTaP vaccine; (C) 2-PE contained in IPV vaccine.

3.1. Calibration curve and linearity of analytical procedure

The calibration curve, using the Symmetry300C18 Waters column, was built using five concentrations of 2- PE in MilliQ Water: 1.100 mg/mL, 0.550 mg/mL, 0.275 mg/mL, 0.137 mg/mL and 0.068 mg/mL. The correlation (r^2) coefficient resulted to be equal to 0.999, indicating a very good proportionality between the 2-PE concentrations and the peak area (Fig. 2A). The RSD % of each point tested in triplicate was 0.48, 0.03, 0.04, 0.03, 0.01, respectively.

The linearity of the analytical procedure, using the Symmetry300C18 Waters column, was determined using five concentrations of 2- PE spiked in a vaccine matrix 2-PE free (HepA): 1.100 mg/mL, 0.550 mg/mL, 0.275 mg/mL, 0.137 mg/mL and 0.068 mg/mL. The correlation (r2) coefficient resulted to be equal to 0.999, indicating a very good proportionality between the 2-PE concentrations in the sample and the peak area (Fig. 2B). The RSD % of each point tested in triplicate was 0.25, 0.37, 0.62, 0.29, 0.11, respectively.

3.2. Accuracy

To investigate this parameter, a 2-PE free vaccine sample (HepA) was spiked with known amounts of 2-PE, in order to evaluate the percentage of recovery. The percentage of recovery was investigated at five

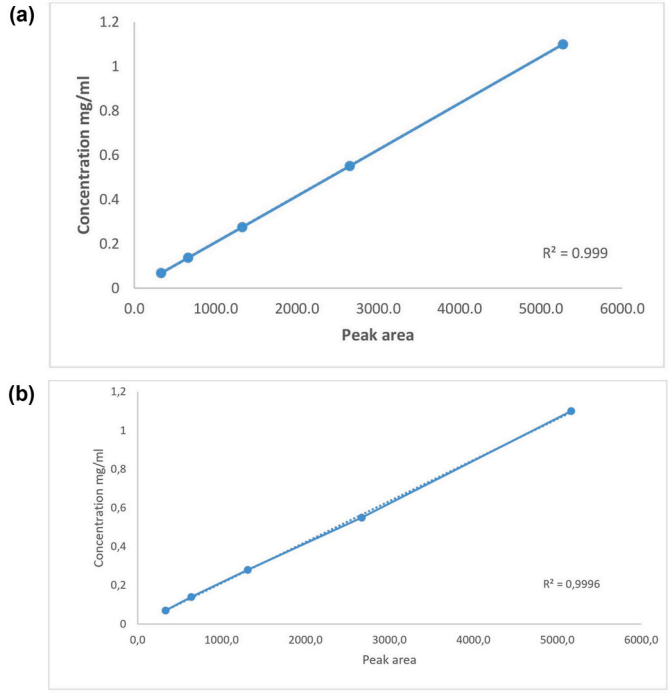

Fig. 2. Calibration curve with 2 –PE standard on Waters Symmetry 300 column (A); linearity of analytical procedures (B).

Table 3
Theoretical concentration, estimated concentration and recovery percentage of 2-PE at five different concentrations.

Theoretical concentration	Estimated concentration	Recovery %
1.10 mg/mL	1.07 mg/mL	97.35%
0.55 mg/mL	0.55 mg/mL	100.60%
0.28 mg/mL	0.27 mg/mL	98.91%
0.14 mg/mL	0.13 mg/mL	96.50%
0.07 mg/mL	0.07 mg/mL	100.03%

different concentrations (Table 3). The highest recovery percentage occurred at 2-PE concentrations of 0.55 mg/mL and 0.07 mg/mL, that is 100.60% and 100.03%, respectively. However, an acceptable recovery was also obtained at the other concentrations tested (1.10 mg/mL, 0.28 mg/mL, 0.14 mg/mL). The method is therefore considered accurate throughout the concentration range of 0.07–1.10 mg/mL.

3.3. Precision

The Precision was considered at two levels:

- Repeatability (intra-analysis precision), intended as the precision in the same operating conditions in a short interval of time;
- Intermediate precision (inter-analysis precision), expressing variations of analysis performed in different days of analysis.

3.3.1. Repeatability

Repeatability was investigated using 6 independent determinations of two vaccines containing a known amount of 2-PE. The 2-PE concentration contained in the tested vaccines, according to the authorized specification, is between 4.4 mg/mL and 6.6 mg/mL. Therefore, in order to operate within the range of linearity and accuracy previously determined (1.1–0.07 mg/mL), the two vaccines were diluted 1:10 to obtain a theoretical 2-PE concentration of 0.44–0.66 mg/mL. The RSD % of the peak area and the concentration resulted very low for both vaccines (IPV-, 0.33 and 0.33; DTaP-IPV, 0.54 and 0.54 respectively), thus supporting the repeatability of the method. Furthermore, the 2-PE concentration in the tested vaccines, was confirmed to comply with the specifications, resulting to be 6.1 mg/mL for the vaccine IPV and 6.2 mg/mL for the vaccine DTaP-IPV.

3.3.2. Intermediate precision

Intermediate precision was investigated on three different days using the same repeatability conditions. The RSD% of the peak area and the concentration resulted very low for both vaccines (IPV 0.79 and 0.35, respectively; DTaP-IPV, 0.98 and 0.32, respectively), thus demonstrating the intermediate precision of the method.

3.4. Robustness

The robustness of the 2-PE quantitation method was evaluated respect to changes of the flow rate, percentage of ACN in mobile phase and column temperature (Table 4).

Table 4
2-PE Concentration, Area, RT and r^2 results at different conditions: (A) Change of column temperature; (B) Change of ACN percentage in mobile phase; (C) Change of flow rate.

	A			B			C		
2 PE Concentration mg/mL	**Area**	**RT**	r^2	**Area**	**RT**	r^2	**Area**	**RT**	r^2
	Temperature 20°C			***47% H2O 53% ACN***			***Flow 0.9 mLl/min***		
1.10	5285.02	1.62	0.999	5156.95	1.67	0.999	5816.07	1.78	0.999
0.55	2663.56	1.62		2585.23	1.67		2956.98	1.78	
0.28	1334.04	1.63		1286.64	1.67		1469.20	1.78	
0.14	666.39	1.63		637.89	1.67		737.88	1.79	
0.07	329.58	1.63		315.36	1.67		365.61	1.79	
Standard Working Conditions	***Temperature 25°C***			***45% H2O 55% ACN***			***Flow 1.0 mL/min***		
1.10	5255.09	1.61	0.999	5185.67	1.63	0.999	5255.09	1.61	0.999
0.55	2648.58	1.61		2575.33	1.63		2648.58	1.61	
0.28	1327.46	1.61		1279.62	1.63		1327.46	1.61	
0.14	662.33	1.61		635.26	1.63		662.33	1.61	
0.07	330.12	1.61		316.36	1.63		330.12	1.61	
	Temperature 30°C			***43% H2O 57% ACN***			***Flow 1.1 mL/min***		
1.10	5220.31	1.60	0.999	5183.77	1.59	0.999	4787.08	1.47	0.999
0.55	2630.37	1.60		2603.52	1.56		2410.66	1.47	
0.28	1316.62	1.59		1295.20	1.56		1217.00	1.47	
0.14	658.14	1.59		641.84	1.56		606.19	1.47	
0.07	327.71	1.59		317.43	1.56		299.98	1.47	

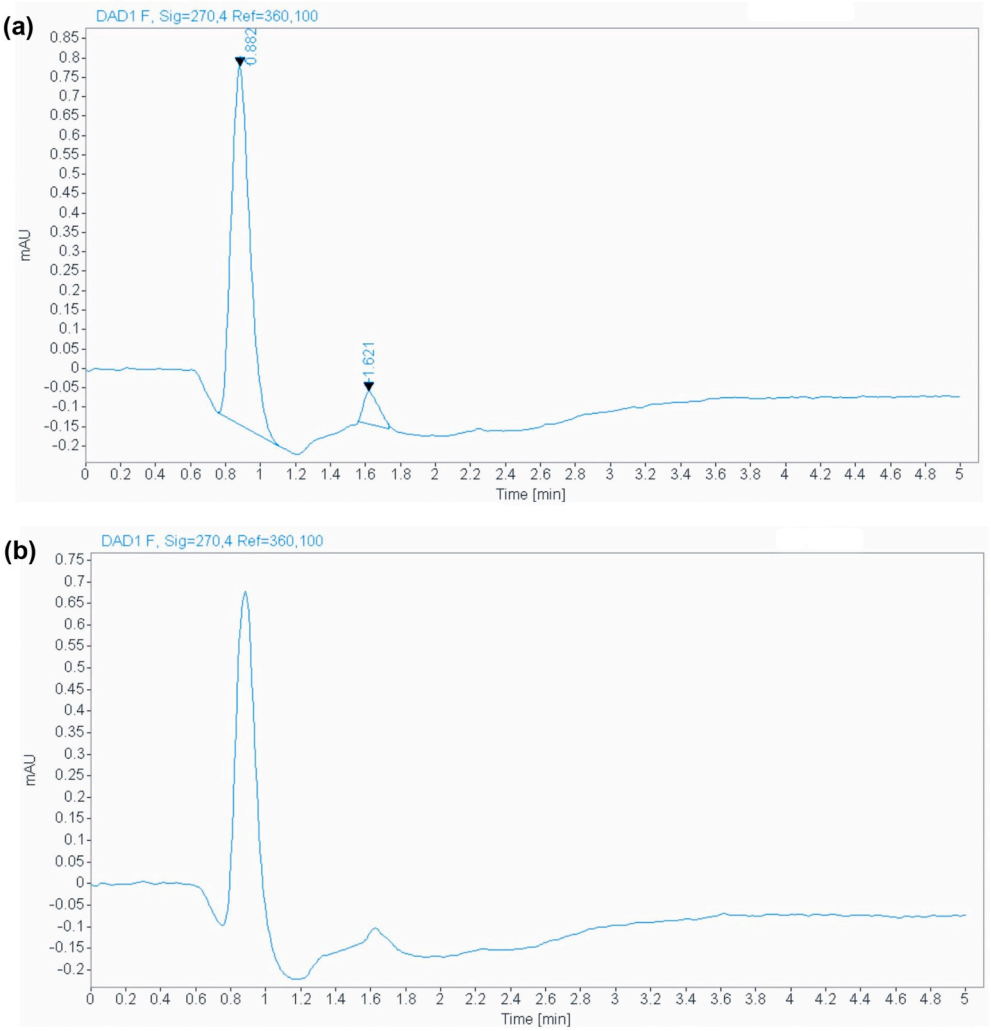

Fig. 3. Chromatogram of one of three independent replicates of the 2-PE concentration 1.3 × 10-4 mg/mL (A) and of the 2-PE concentration 6.7 × 10-5 mg/mL (B).

3.4.1. Change in column temperature

To investigate the entity of temperature impact on the method performance, three runs were performed setting the temperature of the column oven at 20 °C, 25 °C and 30 °C. Three calibration curves were prepared starting from the standard solution of 2-PE, with a concentration of 1.1 to 0.0687 mg/mL and run at the three different temperatures. The correlation coefficient (r^2) remained always above the value 0.995 at the three different conditions, thus demonstrating that the temperature did not impact on the linearity of the method. In order to visually assess the impact of the change in temperatures on the retention time of the 2-PE, the peaks of the standard samples at the concentration of 1.1 mg/mL at the three different temperatures were overlapped. The impact on the RT by the temperature change is negligible.

3.4.2. Variation of ACN percentage in mobile phases

To investigate the impact of the mobile phase composition on the method performance, variation of ±2% ACN in mobile phase were investigated. In particular, three calibration curves were run using three different mobile phases: (i) 45% H_2O and 55% ACN, standard working condition; (ii) 43% H_2O and 57% ACN (+2%) and (iii) 47% H_2O and 53% ACN (−2%). The correlation coefficient (r^2) was above the value 0.995 at the three different condition, thus demonstrating that small differences in the %ACN did not impact on the linearity of the method.

3.4.3. Flow rate variation

To investigate the impact of the flow variations on the robustness of the method, three calibration curves were run at a flow of 1.1 mL/min or 1 mL/min (normal conditions) or 0.9 mL/min, respectively. The linearity and the maintenance of the correlation coefficient (r^2) above the value 0.995 at the three different conditions was evaluated. The results showed that the variation of flow rate induced a minor shift in the retention time, while the correlation coefficient is not affected.

3.5. Detection and quantitation limit

The method described in the present work is not intended to be used as a limit test. For completeness of the validation, LOD and LOQ were determined. In the event that the absence of 2-PE in a given vaccine should be demonstrated, this method could be applied and the LOD should be used as the minimum level of the detectable.2-PE. Therefore, the detection and quantification limits of the proposed method have been both determined by visual evaluation according to the indications of the ICH Q2 guideline for instrumental methods [7].

3.5.1. Detection limit

Serial 1:2 dilutions of a 2-PE free vaccine, spiked with known amounts of 2-PE standard, were prepared. Starting from the concentration of 1.1 mg/mL, dilutions were made up to a concentration of 2.0 × 10^{-6} mg/mL. The chromatograms were checked visually for the

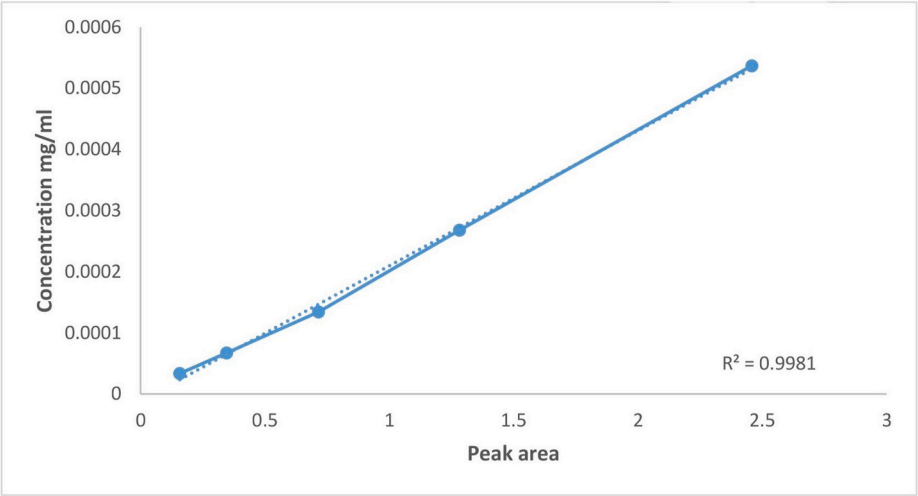

Fig. 4. 2-PE calibration curve for the LOQ determination.

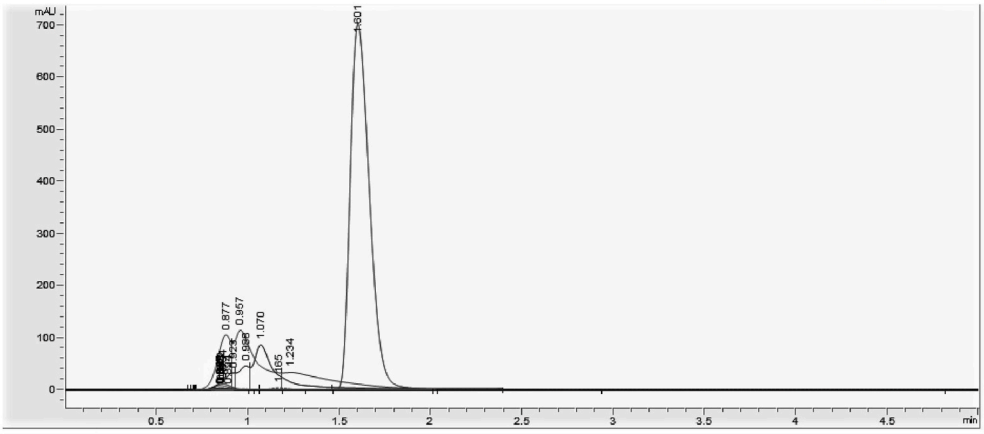

Fig. 5. Evaluation of the specificity of the method using 2-PE free vaccines: overlay of chromatograms of 2-PE free vaccines and 2-PE standard.

presence of 2-PE. The detection limit resulted 1.3×10^{-4} mg/mL (Fig. 3A). At the concentration, 6.7×10^{-5} mg/mL, the peak was still visible, but the identification was not reliable as the height of the peak would be easily confused with background noise and could not be appropriately integrated (Fig. 3B).

3.5.2. *Quantitation limit*

Limit of quantitation was established by using two fold dilutions of a 2-PE free vaccine, spiked with known amounts of 2-PE standard. A standard curve at low 2-PE concentrations ranging from 6.75×10^{-5} to 1.08×10^{-3} mg/mL was prepared. A very high r^2 was obtained at such low 2-PE concentration (Fig. 4). The concentration of 2.7×10^{-4} mg/mL was identified as LOQ, being the lowest amount with a repeatability (RSD%: 4.63) and an accuracy (recovery: 101.51%) in line with the predetermined acceptance criteria (RSD% <5%, Recovery% 100 ± 20%).

3.6. *Specificity and identity*

The specificity and identity were evaluated by analyzing vaccines with and without 2-PE and comparing the chromatographic profile with the 2-PE standard.

The identity was determined by calculating the ratio between the RT of the 2-PE in the standard and in the vaccine samples containing 2-PE. The 2-PE RT peak of the standard and the vaccine samples overlapped: 1.603 min for the 2-PE in the vaccine sample and 1.601 min for the standard. The ratio obtained was 0.998 for both vaccine, thus complying with the validation acceptance criteria.

The specificity requirement was the absence of 2-PE peak in samples of 2-PE free vaccines. To this end, vaccine with different excipients (Table 1) were used. The method was shown to discriminate 2-PE from other substances present in the formulated vaccine (Fig. 5).

4. Discussion

As a best practice, the new RP-UHPLC method was validated according to ICH Q2 Validation of analytical procedures: text and methodology [7]. The European Pharmacopoeia indicate a method for the quantification of phenol in vaccines [8], but no method is described for the determination of 2-PE. Thus the method was developed to establish the presence of 2-PE in commercially available vaccines, and to measure its contents if necessary. The only available study on the quantification of 2-PE in vaccine was performed several years ago by using a RP-HPLC on only one type of vaccine constituted by three antigens, ie. diphtheria, tetanus and whole pertussis [6].

In contrast, in this study the detection of 2-PE was investigated in vaccines, whether containing the preservative or spiked with it, much more complex in terms of antigens and excipients. The pretreatment of the vaccine by centrifugation is important as it drastically reduces the presence of antigens and adjuvant in the supernatant that is injected into

the chromatographic column.

The accuracy determination in a different matrix (HepA vaccine, without 2 PE) has been performed to demonstrate the absence of formulation interfering effects. A good accuracy result (Table 3) has been interpreted as a demonstration of the method suitability for 2 PE determination in that given formulation. When applying this method to vaccine samples with formulations different from those tested in the present study, the above described approach (accuracy determination) should be applied to demonstrate the method suitability. The resulting method is robust and accurate as well as rapid as one run last only 5 min. Furthermore, the mobile phase, which consists of only water/acetonitrile (45/55), is much simpler compared with that of the RP-HPLC study of Bipin Sharma et al. [6], which consisted of tetrahydrofuran/water/methanol/acetonitrile (5/60/10/25). Therefore, an important advantage of this method is the consumption of a very limited amount of reagents and thus also the production of a low amount of waste and consequently an overall reduction of cost.

5. Conclusions

The developed chromatographic method, using the reverse-phase column, Waters Symmetry300C18 with UV spectrophotometric detection, was successfully applied to quantify the 2-PE content in vaccines for human use. The validation results showed that the method is linear, accurate, precise, specific and robust. The matrix, as well as the different antigens present in the vaccine do not interfere with the 2-PE determination. The procedure described is simple and rapid. Thus, the developed method is considered suitable to measure 2-PE content and to assess the eventual presence of 2-PE in marketed vaccines, in order to verify compliance to the approved release specifications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biologicals.2020.12.005.

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