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ORIGINAL RESEARCH ARTICLE

Comparative study on the determination of free formaldehyde residue in vaccines by pre-column derivative liquid chromatography and post-column derivatization liquid chromatography

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ABSTRACT

Objective: To develop and compare methods for quantifying residual free formaldehyde in vaccines using pre-column and post-column derivatization liquid chromatography, and to assess the consistency between these methods. **Methods:** For pre-column derivatization, we used a Shimadzu LC-20AT liquid chromatograph equipped with an SPD-20A UV detector. Separation was achieved with a mobile phase consisting of 60% acetonitrile at a flow rate of 0. 8 $mL \cdot min^{-1}$ and a column temperature of 40 ℃. Detection was performed at 360 nm using a Kromasil 100-5-C_{18} (250 mm × 4.6 mm) column. For post-column derivatization, we employed a Shimadzu LC-20AT liquid chromatograph with an SPD-M20A diode array detector and a vector derivative device. Separation was conducted with a mobile phase of 0.2% (V/V) phosphoric acid at a flow rate of 1.0 $mL \cdot min^{-1}$ and a column temperature of 25 ℃. Detection was carried out at 412 nm using a Chrom Core AQ-C_{18} (250 mm × 4.6 mm) column. The derivatization solution was acetate buffer, flowing at 0.5 $mL \cdot min^{-1}$ and maintained at 100 ℃. Both methods were evaluated for precision, repeatability, and sample recovery, with statistical significance assessed using *F*-test and *t*-test. **Results:** The pre-column derivatization method exhibited linearity over the range of 0.025–100 $\mu g \cdot mL^{-1}$ (R = 0.9999, n = 12). Precision showed an RSD value of 0.06%, and repeatability ranged from 0.3% to 1.4%. Average recoveries were between 97.3% and 104.8%, with RSD values from 0.7% to 2.9%. The limit of quantitation (LOQ) was 0.02 $\mu g \cdot mL^{-1}$, and the limit of detection (LOD) was 0.01 $\mu g \cdot mL^{-1}$. The post-column derivatization method demonstrated linearity in the same range (0.025–100 $\mu g \cdot mL^{-1}$, R = 0.9999, n = 12). Precision showed an RSD value of 0.02%, and repeatability ranged from 0.7% to 3.5%. Average recoveries were between 105.6% and 114.6%, with RSD values from 0.3% to 1.9%. The LOQ was 0.02 $\mu g \cdot mL^{-1}$, and the LOD was 0.006 $\mu g \cdot mL^{-1}$. Statistical analysis indicated that both methods produced comparable results. **Conclusion:** Both pre-column and post-column derivatization liquid chromatography methods are simple, accurate, and highly sensitive, making them suitable for determining residual free formaldehyde in vaccines.

Keywords: vaccine; free formaldehyde; pre-column derivatization liquid chromatography; post-column derivatization liquid chromatographyy

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Inactivated vaccines, such as the novel coronavirus inactivated vaccine (Vero cells). Recombinant hepatitis B vaccine (Saccharomyces cerevisiae) is widely used in people's vaccination. Inactivation step is one of the most critical and basic technologies in vaccine production requiring microbial inactivation, which can prevent the production of pathogens and improve safety at the same time. Using formaldehyde as an inactivator is one of the first important methods to inactivate biological products. As early as 1911, formaldehyde was used to inactivate tetanus toxin to produce toxoid[1]. Recent studies have found that formaldehyde can induce deamination of proteins when it reacts with proteins. Formation α,β-Unsaturated aldehydes and methylation and formylation on two adjacent lysine residues, etc., thus denaturing the protein[2].

Studies at home and abroad have found that formaldehyde not only has eye. Nasal and laryngeal irritation can cause respiratory diseases. Acute toxicity such as acute inflammation and allergy, as well as reproductive and developmental toxicity. Neurotoxicity. Immunotoxicity. Genotoxicity and carcinogenicity[3,4]. The international organization for research on cancer (IARC) under the World Health Organization (who) has listed formaldehyde as a class 1 carcinogen[5]. Therefore, formaldehyde residues in vaccines should be strictly controlled.

According to the requirements of the notice on improving the national drug standards in 2019 (Pharmacopoeia synthesis [2019] No. 35), our institute undertook the drafting task of the standard preparation and revision project of "research and establishment of alternative methods for the determination of vaccine inactivator (free formaldehyde)" (No. 2019$s_1$3), and the rechecking unit was the China Institute for food and drug control[6].

According to the general rule 3207 (determination of free formaldehyde) of the 2020 edition of the Pharmacopoeia of the people's Republic of China (hereinafter referred to as "Chinese Pharmacopoeia"), the content of free formaldehyde in the sample is determined by magenta sulphurous acid colorimetry or acetylacetone colorimetry. About 44% (24) of the 54 preventive vaccines contained in the third volume of the 2020 edition of the Chinese Pharmacopoeia need to determine the residue of free formaldehyde, most of which use fuchsin sulphurous acid colorimetry[7]. At present, the technical specifications for the safety of cosmetics (2015 Edition) stipulates that acetylacetone spectrophotometry and precolumn derivatization liquid chromatography are used to determine the content of formaldehyde in cosmetics[8]; annex 2 of Circular No. 12 of the State Drug Administration in 2019 (Circular on incorporating nine inspection methods such as the detection method of free formaldehyde in cosmetics into the safety technical specifications for cosmetics (2015 version)) stipulates that the determination of water agents is carried out by post column derivatization liquid chromatography. Cream lotion. Free formaldehyde content in gel cosmetics[9]. Determination of formaldehyde content by pre column derivatization liquid chromatography is to use formaldehyde to react with 2,4-dinitrophenylhydrazine to produce yellow formaldehyde 2,4-dinitrophenylhydrazone, and then use liquid chromatography to determine the peak area of formaldehyde 2,4-dinitrophenylhydrazone, so as to obtain the formaldehyde content; the determination of formaldehyde content by post column derivatization liquid chromatography is to use C_{18} chromatographic column to separate the free formaldehyde in the sample. In the post column derivatization device, formaldehyde reacts with acetylacetone and ammonium acetate to produce a yellow derivative product [3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (DDL)]. The absorbance of the product at the wavelength of 412 nm is directly proportional to the content of formaldehyde. Fuchsin sulphurous acid colorimetry and acetylacetone colorimetry are not specific, and are easily disturbed by vaccine matrix and environment; chromatography has the advantages of strong separation ability, high selectivity, high accuracy and sensitivity, and simple operation. In this study, referring to the above industry standards and relevant literature, pre column derivatization liquid chromatography and post column derivatization liquid chromatography were used to determine the residual amount of free formaldehyde in vaccines. The method is

accurate and reliable.

1. Instruments and materials

1.1. Instrument

Shimadzu LC-20AT liquid chromatograph (SPD-20A UV detector, empower 3 processing software) (Shimadzu company of Japan); shimadzu LC-20AT liquid chromatograph (SPD-M20A diode array detector and vector derivative device) (Shimadzu company of Japan); TW20 constant temperature water bath box (julabo company, USA).

1.2. Materials

Formaldehyde solution reference material in water (China Academy of metrology, batch No.: 19001, content 10.1 $mg \cdot mL^{-1}$); 2,4-dinitrophenylhydrazine (mclean, batch No.:C10801329, content 98%). Acetonitrile is chromatographically pure, phosphoric acid. Ammonium acetate. Glacial acetic acid and acetylacetone are both analytically pure.

Sample: a) Recombinant hepatitis B vaccine (Saccharomyces cerevisiae): enterprise a, batch number: A1~A6, except that A1 is 10 $\mu g \cdot 0.5\ mL^{-1} \cdot piece^{-1}$, A3 is 60 $\mu g \cdot 0.5\ mL^{-1} \cdot piece^{-1}$, the specifications of other batches are 20 $\mu g \cdot 0.5\ mL^{-1} \cdot piece^{-1}$. b) Adsorbed acellular DPT combined vaccine: enterprise B, batch number B1~B6, specification 0.5 $mL \cdot bottle^{-1}$. c) Influenza virus split vaccine: enterprise C, batch number: C1~C3, specification: 0.5 $mL \cdot piece^{-1}$; enterprise D, batch number: D1~D3, except that D_3 specification is 0.25 $mL \cdot piece^{-1}$, other specifications are 0.50 $mL \cdot piece^{-1}$. d) Inactivated hepatitis A vaccine (human diploid cells): Enterprise E, batch number E1~E3, specification 0.5 mL.

2. Methods and results

2.1. Chromatographic conditions

2.1.1. Precolumn derivatization liquid chromatography

Shimadzu LC-20at liquid chromatograph (SPD-20A UV detector), using kromasil 100-5-C_{18} (250 mm × 4.6 mm) chromatographic column. The mobile phase is 60% acetonitrile solution, the flow rate is 0.8 $mL \cdot min^{-1}$, the column temperature is 40 ℃, the determination wavelength is 360 nm, and the injection volume is 5 μL.

2.1.2. Post column derivatization liquid chromatography

Shimadzu LC-20AT liquid chromatograph (SPD-M20A diode array detector and vector derivation device), nano spectrum AQ-C_{18} (250 mm × 4.6 mm) chromatographic column. The mobile phase is 0.2% (v/v) phosphoric acid solution, the flow rate is 1.0 $mL \cdot min^{-1}$, the column temperature is 25 ℃, the detection wavelength is 412 nm, and the injection volume is 10 μL; the flow rate of derivative solution is 0.5 $mL \cdot min^{-1}$, and the temperature is 100 ℃.

2.2. Preparation of solution

2.2.1. Buffer solution

Take 32.2 mL of 1 $mol \cdot L^{-1}$ sodium hydroxide solution and 2.8 mL of glacial acetic acid, and dilute to 500 mL with water.

2.2.2. 2,4-dinitrophenylhydrazine acetonitrile solution

Take 150 mg of 2,4-dinitrophenylhydrazine and dilute it to 100 mL with acetonitrile.

2.2.3. Derivative solution

Take 62.5 g ammonium acetate, add 7.5 mL glacial acetic acid and 5 mL acetylacetone, add water to 1000 mL, and shake well. Used within 3D.

2.2.4. Standard stock solution

Accurately measure an appropriate amount of formaldehyde solution reference material in the water, and dilute it with water to make a solution containing 2.02 mg formaldehyde per 1 mL.

2.2.5. Standard solution

Precisely measure an appropriate amount of the standard stock solution and dilute it with water to make solutions containing 0.025, 0.05, 0.10, 0.50, 1, 2, 5, 10, 20, 50, 75 and 100 μg of formaldehyde per 1 mL, respectively.

2.2.6. Preparation of test solution

Precolumn derivatization liquid chromatography:

Take samples.

Post column derivatization liquid chromatography:

A proper amount of sample is centrifuged at 4000 $R \cdot min^{-1}$ for 10 min, 1 mL of supernatant is accurately measured, 1 mL of mobile phase is added, and then mixed.

2.2.7. Blank excipient solution

Prepare blank excipient solution according to the prescription provided by each enterprise.

2.3. Determination method

Pre column derivatization liquid chromatography was used to accurately measure the standard solution respectively. Test solution. Put 0.5 mL of buffer solution and blank excipient solution into a centrifuge tube, add 0.25 mL of buffer solution and 0.5 mL of 2,4-dinitrophenylhydrazine acetonitrile solution, mix well, keep warm in a 60 ℃ water bath for 20 min, cool to room temperature, and filter.

2.4. Methodological validation

2.4.1. Specificity test

Precolumn derivatization liquid chromatography. Take the buffer solution and blank excipient solution, prepare them according to the "2.3" determination method, inject samples according to the "2.1.1" chromatographic conditions, record the chromatogram, and compare them with the standard chromatogram and the test sample chromatogram. The results show that the solvent and excipients do not interfere with the sample test, as shown in **Figure 1**.

Post column derivatization liquid chromatography. Take the mobile phase and blank excipient solution, inject the sample according to the chromatographic conditions of "2.1.2", record the chromatogram, and compare it with the chromatogram of the standard and the chromatogram of the test sample. The results show that the solvent and excipients do not interfere with the sample test, as shown in **Figure 2**.

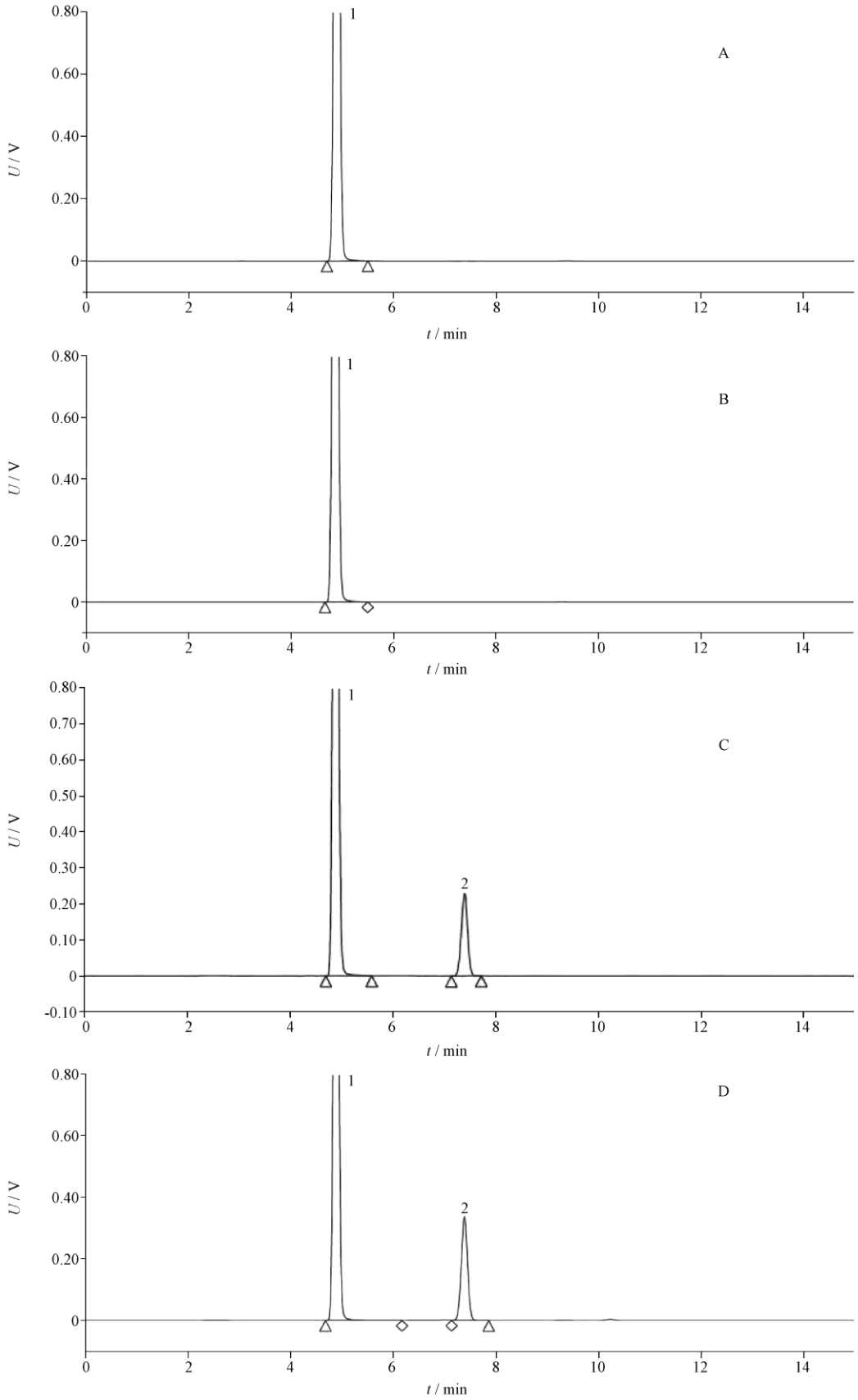

Figure 1. LC chromatograms of pre-column derivatization chromatography. **(A)** buffer solution; **(B)** excipient solution; **(C)** standard solution; **(D)** test sample (lot No.: D2) 1) 2,4-dinitrophenylhydrazine; 2) formaldehyde, 2-(2,4-dinitrophenyl) hydrazonene.

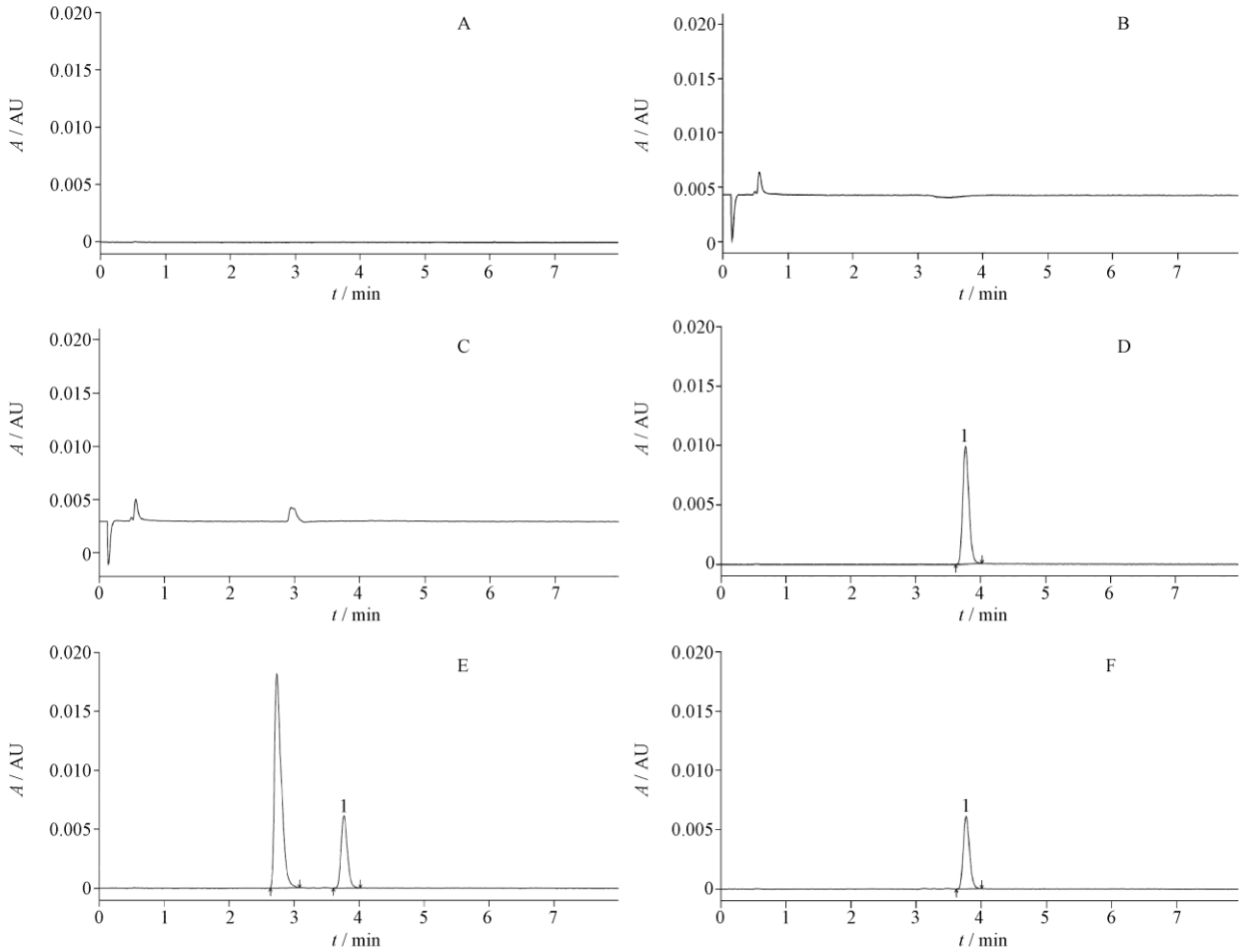

Figure 2. Post-column derivatization chromatography. **(A)** mobile phase; **(B)** excipient solution; **(C)** aluminum adjuvant; **(D)** standard solution; **(E)** test sample of spiked recovery of recombinant hepatitis B vaccine (Saccharomyces cerevisiae (lot No.: A3), not centrifugal; **(F)** test sample of spiked recovery of recombinant hepatitis B vaccine (Saccharomyces cerevisiae (lot No.: A3), centrifugal] 3,5-diacetyl-1,4-dihydro-2,6-lutidine, DDL.

2.4.2. System suitability test

Precolumn derivatization liquid chromatography:

Take the standard solution (20) under "2.2.5" μg·mL^{-1}), prepared according to the "2.3" determination method, inject samples according to the "2.1.1" chromatographic conditions, and record the chromatogram. In the chromatogram of standard solution, the theoretical plate numbers of 2,4-dinitrophenylhydrazine peak and formaldehyde 2,4-dinitrophenylhydrazone peak are 9751 and 10,142 respectively, and the resolution is 10.22, as shown in **Figure 1**.

Post column derivatization liquid chromatography:

Take the standard solution (20) under "2.2.5" μg·mL^{-1}), inject samples according to the chromatographic conditions of "2.1.2", and record the chromatogram. In the chromatogram of standard solution, the theoretical plate number of 3,5-diacetyl-1,4-dihydro-2,6-dimethylpyridine (DDL) peak is 5778, as shown in **Figure 2**.

2.4.3. Investigation of linear relationship

Precolumn derivatization liquid chromatography:

Take the standard solution under "2.2.5", prepare it according to "2.3" determination method, inject the sample according to "2.1.1" chromatographic conditions, and record the chromatogram. Based on the mass

concentration of formaldehyde (C, μg·mL^{-1}) is the abscissa, and the peak area (A) is the ordinate for linear regression. The regression equation is A = 95,757C − 7516, and the correlation coefficient is R = 0.9999 (n = 12).

Post column derivatization liquid chromatography:

Take the standard solution under "2.2.5", inject the sample according to the chromatographic conditions of "2.1.2", and record the chromatogram. Based on the mass concentration of formaldehyde (C, μg·mL^{-1}) is the abscissa, and the peak area (A) is the ordinate for linear regression. The regression equation is A = 56,464C + 1324, and the correlation coefficient is R = 0.9999 (n = 12).

2.4.4. Inspection of quantitation limit and detection limit

Precolumn derivatization liquid chromatography:

Take the standard solution (0.025) under "2.2.5 μg·mL^{-1}), dilute step by step, prepare according to the "2.3" determination method, inject samples according to the "2.1.1" chromatographic conditions, and record the chromatogram. The quantitation limit is measured when S/N = 10 and the detection limit is measured when S/N = 3. The quantitative limit of formaldehyde is 0.02 μg·mL^{-1}, detection limit is 0.01 μg·mL^{-1}.

Post column derivatization liquid chromatography:

Take the standard solution (0.025) under "2.2.5 μg·mL^{-1}), dilute step by step, inject the sample according to the chromatographic conditions of "2.1.2", and record the chromatogram. The quantitation limit is measured when S/N = 10 and the detection limit is measured when S/N = 3. The quantitative limit of formaldehyde is 0.02 μg·mL^{-1}, detection limit is 0.006 μg·mL^{-1}.

2.4.5. Precision test

Precolumn derivatization liquid chromatography:

Take the standard solution (20) under "2.2.5" μg·mL^{-1}), prepared according to the "2.3" determination method, inject samples continuously for 6 times according to the "2.1.1" chromatographic conditions, and record the chromatogram. The RSD of peak area is 0.06%.

Post column derivatization liquid chromatography:

Take the standard solution (20) under "2.2.5" μg·mL^{-1}), inject six consecutive times according to the chromatographic conditions of "2.1.2", and record the chromatogram. The RSD of peak area is 0.02% (n = 6).

2.4.6. Repeatability test

Precolumn derivatization liquid chromatography:

Sample 4 batches, prepare 6 solutions for each batch according to the "2.3" determination method, inject samples according to the "2.1.1" chromatographic conditions, record the chromatogram and calculate. The results are shown in **Table 1**.

Post column derivatization liquid chromatography:

Take 4 batches of samples, prepare 6 test sample solutions according to the method of "2.2.6.2", inject samples according to the chromatographic conditions of "2.1.2", record the chromatogram and calculate. The results are shown in **Table 1**.

Table 1. Repeatability test results.

Product	Pre-column derivatization chromatography			Post-column derivatization chromatography		
	Batch No.	Formaldehyde content/(μg·mL^{-1})	RSD/%	Batch No.	Formaldehyde content)/(μg·mL^{-1})	RSD/%
Recombinant hepatitis B vaccine (Saccharomyces cerevisiae	A6	1.1	0.3	A_5	1.0	0.6
Diphtheria, tetanus and acellular pertussis combined vaccine, adsorbed	B6	25.0	1.4	B_3	24.1	0.07
Influenza vaccine (split virion), inactivated	D2	30.1	0. 9	D_1	30.2	1.1
Hepatitis A vaccine (human diploid cell), inactivated	E3	0.09	1.3	E_2	0.08	3.5

2.4.7. Intermediate precision test

Precolumn derivatization liquid chromatography:

Different testers use different instruments (Shimadzu LC-20at liquid chromatograph and waterse$_2$695 liquid chromatograph) at different times to take 4 batches of standard solution and samples, prepare them according to the "2.3" determination method, inject samples according to the "2.1.1" chromatographic conditions, record the chromatogram and calculate. The intermediate precision RSD% is less than 3.0.

Post column derivatization liquid chromatography:

Four batches of samples are sampled by different test personnel at different times, and the test solution is prepared according to the method of "2.2.6.2". The samples are injected according to the chromatographic conditions of "2.2.1", and the chromatogram is recorded and calculated. The intermediate precision RSD% is less than 2.0.

2.4.8. Recovery rate test

Precolumn derivatization liquid chromatography.

a) Recombinant hepatitis B vaccine (saccharomyces cerevisiae): Precisely measure samples (batch number A3, formaldehyde residue is 1.29 μg·mL^{-1}) 0.25 mL, put them in 9 centrifuge tubes, each 3 parts are a group, each add standard solution (5.0 μg·mL^{-1}) 0.05, 0.06, 0.08 mL and water 0.20, 0.19, 0.17 mL to each group respectively, prepare according to "2.3" determination method, inject samples according to "2.1.1" chromatographic conditions, and record the chromatogram figure, calculated recovery results, see **Table 2**.

Table 2. Results of spiked recovery of recombinant hepatitis B vaccine (Saccharomyces cerevisiae).

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
0.322	0.253	0.269	106.4	104.8	2.0
0.322	0.253	0.271	107.2		
0.322	0.253	0.272	107.6		
0.322	0.303	0.318	105.0		
0.322	0.303	0.318	104.9		
0.322	0.303	0.318	105.1		
0.322	0.404	0.411	101.6		
0.322	0.404	0.415	102.7		
0.322	0.404	0.416	102.9		

b) Adsorbed acellular DTP combined vaccine: Precisely measure 0.25 mL of the test sample (batch number is B6, formaldehyde residue is 24.73 μg·mL^{-1}), and put them in 9 centrifuge tubes, and each 3 parts is a group. Add standard solution (100 μg·mL^{-1}) 0.048, 0.060, 0.072 mL and water 0.202, 0.190, 0.178 mL to each group respectively, prepare according to "2.3" determination method, inject samples according to "2.1.1" chromatographic conditions, record the chromatogram figure, calculated recovery results, see **Table 3**.

Table 3. Results of spiked recovery of diphtheria, tetanus and acellular pertussis combined vaccine, adsorbed.

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
6.183	4.848	4.599	94.9	97.3	1.8
6.183	4.848	4.663	96.2		
6.183	4.848	4.616	95.2		
6.183	6.060	5.885	97.1		
6.183	6.060	5.888	97.2		
6.183	6.060	5.878	97.0		
6.183	7.272	7.202	99.0		
6.183	7.272	7.218	99.3		
6.183	7.272	7.254	99.8		

c) Influenza virus split vaccine: Precisely measure 0.25 mL of the test sample (batch number is D2, formaldehyde residue is 29.44 μg·mL^{-1}), put it in 9 centrifuge tubes, and each 3 parts is a group, and each group is added separately. Standard solution (100 μg·mL^{-1}) 0.060, 0.075, 0.090 mL and water 0.190, 0.175, 0.160 mL, prepared according to the "2.3" assay method, injected according to the "2.1.1" chromatographic conditions, record the chromatogram, and calculate the recovery. The results can be seen in **Table 4**.

Table 4. Results of spiked recovery of influenza vaccine (split virion), inactivated.

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
7.360	6.060	6.281	103.7	103.8	0.7
7.360	6.060	6.328	104.4		
7.360	6.060	6.280	103.6		
7.360	7.575	7.859	103.8		
7.360	7.575	7.982	105.4		
7.360	7.575	7.860	103.8		
7.360	9.090	9.384	103.2		
7.360	9.090	9.403	103.4		
7.360	9.090	9.354	102.9		

d) Inactivated hepatitis A vaccine (human diploid cells): accurately measure the test sample (batch number E_3, formaldehyde residue 0.08 μg·mL^{-1}) 0.25 mL, put into 6 centrifuge tubes, and add standard solution (0.50) respectively μg·mL^{-1}) 0.04 mL and 0.21 mL of water, prepared according to the "2.3" determination method, injected according to the "2.1.1" chromatographic conditions, recorded the chromatogram, and calculated the recovery results, as shown in **Table 5**.

Table 5. Results of spiked recovery of hepatitis *A* vaccine (human diploid cell), inactivated.

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
0.021	0.020	0.022	107.5	103.6	2.9
0.021	0.020	0.020	99.3		
0.021	0.020	0.020	100.9		
0.021	0.020	0.021	104.9		
0.021	0.020	0.021	104.5		
0.021	0.020	0.021	104.4		

a) Post column derivatization liquid chromatography:

Recombinant hepatitis B vaccine (saccharomyces cerevisiae): Take a sample (batch number A3, formaldehyde residue is 1.27 $\mu g \cdot mL^{-1}$), centrifuge at 4000 $r \cdot min^{-1}$ for 10 min, respectively accurately measure 0.25 mL of the supernatant, put it in the in 9 centrifuge tubes, each of 3 parts is a group. Add standard solution (5.0 $\mu g \cdot mL^{-1}$) 0.05, 0.06, 0.08 mL, mobile phase 0.20, 0.19, 0.17 mL to each group, mix well, press "2.1.2" chromatographic conditions were injected, the chromatogram was recorded, and the recovery results were calculated. See **Table 6**.

Table 6. Results of spiked recovery of recombinant hepatitis B vaccine (Saccharomyces cerevisiae).

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
0.318	0.253	0.276	109.25	107.8	1.0
0.318	0.253	0.274	108.55		
0.318	0.253	0.274	108.71		
0.318	0.303	0.328	108.29		
0.318	0.303	0.329	108.62		
0.318	0.303	0.325	107.30		
0.318	0.404	0.432	106.88		
0.318	0.404	0.431	106.57		
0.318	0.404	0.430	106.39		

b) Adsorption of acellular DTP combined vaccine: take a sample (batch number is B5, formaldehyde residue is 25.84 $\mu g \cdot mL^{-1}$), centrifuge at 4000 $r \cdot min^{-1}$ for 10 min, and accurately measure 0.25 mL of the supernatant, set at 9 in each centrifuge tube, each 3 parts is a group. Add standard solution (100 $\mu g \cdot mL^{-1}$) 0.048, 0.060, 0.072 mL, mobile phase 0.202, 0.190, 0.178 mL to each group, mix well, press "2.1.2" "The chromatographic conditions were injected, the chromatogram was recorded, and the recovery results were calculated. See **Table 7**.

Table 7. Results of spiked recovery of diphtheria, tetanus and acellular pertussis combined vaccine, adsorbed.

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
6.460	4.848	5.122	105.65	105.6	0.3
6.460	4.848	5.139	106.00		
6.460	4.848	5.140	106.03		
6.460	6.060	6.384	105.34		
6.460	6.060	6.406	105.71		
6.460	6.060	6.412	105.81		

6.460	7.272	7.653	105.24
6.460	7.272	7.675	105.55
6.460	7.272	7.668	105.44

c) Influenza virus split vaccine: Take the sample (batch number is D2, the formaldehyde residue is 31.99 μg·mL^{-1}), centrifuge at 4000 r·min^{-1} for 10 min, and accurately measure 0.25 mL of the supernatant, respectively, and place 9 centrifuge tubes, each 3 parts is a group, and each group is added with standard solution (100 μg·mL^{-1}) 0.060, 0.075, 0.090 mL, mobile phase 0.190, 0.175, 0.160 mL, mix well, according to "2.1.2" chromatographic conditions Inject the sample, record the chromatogram, and calculate the recovery results. See **Table 8**.

Table 8. Results of spiked recovery of influenza vaccine (split virion), inactivated.

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
7.998	6.06	6.725	110.97	111.0	0.4
7.998	6.06	6.734	111.12		
7.998	6.06	6.745	111.31		
7.998	7.58	8.420	111.15		
7.998	7.58	8.448	111.53		
7.998	7.58	8.445	111.49		
7.998	9.09	10.021	110.24		
7.998	9.09	10.038	110.43		
7.998	9.09	10.047	110.53		

d) Inactivated hepatitis A vaccine (human diploid cells): Take the sample (batch number is E2, the formaldehyde residue is 0.076 μg·mL^{-1}), centrifuge at 4000 r·min^{-1} for 10 min, and accurately measure the supernatant respectively. 0.25 mL of the solution was placed in 6 centrifuge tubes, and 0.04 mL of the standard solution (0.50 μg·mL^{-1}) and 0.21 mL of the mobile phase were added, and the mixture was mixed. Calculate the recovery results. See **Table 9**.

Table 9. Results of spiked recovery of hepatitis *A* vaccine (human diploid cell), inactivated.

Originalamount/μg	Added/μg	Tested/μg	Recovery/%	Average recovery/%	RSD/%
0.019	0.0202	0.023	114.95	114.6	1.9
0.019	0.0202	0.023	112.47		
0.019	0.0202	0.023	113.22		
0.019	0.0202	0.023	112.99		
0.019	0.0202	0.023	116.23		
0.019	0.0202	0.024	117.88		

2.4.9. Scope

Range refers to the precision of the analytical method. When accuracy and linearity are required, the range of high and low limit concentrations or quantities usually needs to meet the minimum concentration level of the coverage method. Pay attention to the concentration level. Accuracy and precision of high concentration level. In this test, the quantitative limits of pre column derivatization liquid chromatography and post column derivatization liquid chromatography are both 0.02 μg·mL^{-1}, linear range 0.025~100 μg·mL^{-1}, the sample concentration of repeatability test is 0.08~30.20 μg·mL^{-1}, the recovery test concentration is 0.08~32.4

μg·mL^{-1}. Therefore, the range of both methods is 0.02~100 μg·mL^{-1}.

2.4.10. Sample determination

Precolumn derivatization liquid chromatography:

Take samples, prepare according to the "2.3" determination method, inject samples according to the "2.1.1" chromatographic conditions, and record the chromatogram. The corresponding peak area is regressed by the concentration of the standard solution, and the measured peak area of the test solution is substituted into the linear regression equation to calculate the formaldehyde residue in the test solution. The results are shown in **Tables 10** and **11**.

Table 10. Results of samples (diphtheria, tetanus and acellular pertussis combined vaccine, adsorbed and influenza vaccine (split virion), inactivated).

Product	**Manufacturer**	**Batch No.**	**Formaldehyde/(μg·mL^{-1})**		
			Pre-column derivatization chromatography)	**Post-column derivatization chromatography**	**Fuchsin-sulfurous acid colorimetry**
Diphtheria, tetanus and acellular Pertussis combined vaccine, adsorbed	B	B_1	24.6	25.0	24
		B_2	24.9	25.0	24
		B_3	24.6	24.1	23
		B_4	24.6	24.8	23
		B_5	25.4	25.3	24
		B_6	25.0	25.4	24
Influenza vaccine (split virion), inactivated	C	C_1	0.3	0.2	3
		C_2	0.3	0.2	3
		C_3	0.2	0.2	2
	D	D_1	29.4	30.2	27
		D_2	30.1	30.2	28
		D_3	30.4	31.5	28

Table 11. Results of sample of [spiked recovery of recombinant hepatitis B vaccine (Saccharomyces cerevisiae) and spiked recovery of hepatitis A vaccine (human diploid cell), inactivated].

Product	**Manufacturer**	**Batch No.**	**Formaldehyde/(μg·mL$^{-1)}$**		
			Pre-column derivatization chromatography)	**Post-column derivatization chromatography**	**Fuchsin-sulfurous acid colorimetry**
Spiked recovery of recombinant hepatitis B Vaccine (Saccharomyces cerevisiae)	A	A1	1.1	1.1	2
		A2	1.2	1.2	2
		A3	1.3	1.3	2
		A4	1.1	1.1	2
		A5	1.1	1.0	2
		A6	1.1	1.1	2
Spiked recovery of hepatitis A vaccine (human diploid cell), inactivated	E	E1	0. 1	0. 07	1
		E2	0. 1	0. 08	1
		E3	0. 09	0. 07	1

Post column derivatization liquid chromatography:

Take samples, prepare according to "2.2.6.2", inject samples according to "2.1.2" chromatographic

conditions, and record the chromatogram. Make a linear regression on the corresponding peak area with the concentration of the standard solution, and substitute the measured peak area of the test solution into the linear regression equation to calculate the formaldehyde residue in the test solution. The results are shown in **Tables 10** and **11**.

2.5. Statistical test of sample determination results

Graphpadprism$_6$.0 software was used to analyze the measurement results of the two methods, and the difference was statistically significant when $P \leq 0.05$. *F* test was used to analyze the overall variance of the data, $P = 0.95$, indicating that there was no significant difference in the variance of the two groups of data. Unpaired *t*-test was used for data analysis, $P = 0.98$, indicating that the difference between the results measured by the two methods was not statistically significant ($P > 0.05$).

2.6. Comparison with the results of the review unit

The China Institute for food and drug control reviewed the standards and samples drafted by our institute, and the sample determination results were consistent with the results of our institute.

3 Discussion

3.1. Precolumn derivatization liquid chromatography

3.1.1. Selection of 2,4-dinitrophenylhydrazine solution concentration

2,4-dinitrophenylhydrazine is slightly soluble in water and ethanol and soluble in acid. When the mobile phase is 60% acetonitrile solution, if 2,4-dinitrophenylhydrazine is dissolved with acid, it is easy to form 2,4-dinitrophenylhydrazine to precipitate after injection, thus blocking the chromatographic column. If acetonitrile is used to dissolve 2,4-dinitrophenylhydrazine, crystallization will occur in the derivation reaction when the concentration is too high. Therefore, 1.5 mg·mL^{-1} 2,4-dinitrophenylhydrazine acetonitrile solution was selected as the derivatization reagent.

3.1.2. Selection of derivative reaction temperature and time

The reaction temperature has a significant effect on the derivation process, and increasing the reaction temperature can shorten the reaction time. It is found that the reaction equilibrium can be achieved only after reacting for 2 h in a 40 ℃ water bath, so the reaction temperature is 60 ℃ and the reaction time is 20 min.

3.1.3. Selection of the proportion of each solution in the derivation reaction

The test found that the test solution. When the reaction volume of buffer solution and 2,4-dinitrophenylhydrazine acetonitrile solution is 0.5 mL, after the derivative reaction, the mixed solution has red crystals after being placed at room temperature (15 ℃), indicating that the reaction system is unstable. The crystal is confirmed to be 2,4-dinitrophenylhydrazine by test. It is speculated that the proportion of aqueous solution in the reaction system is too high, resulting in the precipitation of 2,4-dinitrophenylhydrazine. When the volume of buffer solution was reduced to 0.25 mL, the mixed solution was placed at room temperature (15 ℃) for 16 h without crystallization; the peak area of formaldehyde 2,4-dinitrobenzone did not change significantly after the mixed solution was placed for 2 h and injected every 15 min. Therefore, the test solution is selected. The reaction volumes of buffer solution and 2,4-dinitrophenylhydrazine acetonitrile solution were 0.25 mL and 0.5 mL, respectively.

3.1.4. Sample pretreatment

According to the literature[10], the sample needs to be centrifuged before derivatization. In this experiment, the sample treated by centrifugation and the sample without pretreatment were compared. There was no

significant difference in the determination results after the derivative reaction, indicating that the sample can accurately determine the residue of free formaldehyde without treatment.

3.2. Post column derivatization liquid chromatography

3.2.1. Selection of measurement wavelength

The State Drug Administration Circular No. 12 of 2019 stipulates that the determination wavelength of post column derivatization liquid chromatography is 420 nm[9]. In the test, it was found that the maximum absorption wavelength of DDL peak was 412.8 nm (**Figure 3**), and according to the general rule 3207 (determination of free formaldehyde) acetylacetone colorimetry of Chinese Pharmacopoeia 2020 edition, the absorbance of derivative products was measured at the wavelength of 412 nm[7]. Therefore, 412 nm is selected as the measurement wavelength.

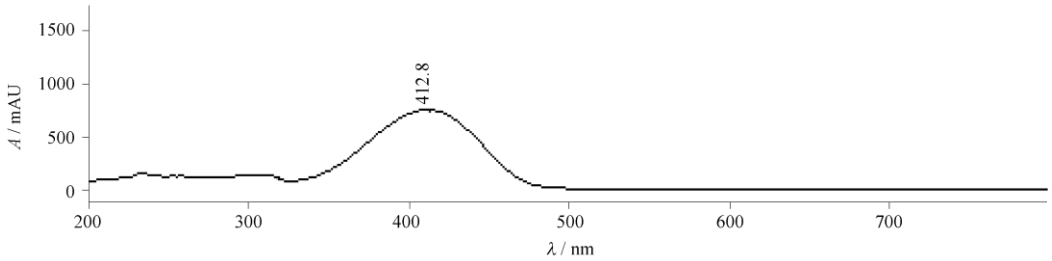

Figure 3. Scanning results of maximum absorption wavelength.

3.2.2. Selection of derivative reagent flow rate and derivative temperature

Take the standard solution (1 μg·mL^{-1}), and the effects of the flow rate of the post column derivatization reagent and the derivatization temperature on the peak area of the derivates were investigated. It was found that the peak area of DDL increased with the increase of the flow rate of derivative reagent; the peak area of DDL increased with the increase of derivation temperature. Considering two factors, the flow rate of derivative reagent is 0.5 mL·min^{-1}, and the derivative temperature is 100 ℃.

3.2.3. Sample pretreatment

It was found that the aluminum adjuvant and the antigen combined with the aluminum adjuvant would react with the derivative reagent, and there were heteropeaks in the chromatogram of the tested product (**Figure 2**). It is speculated that aluminum can react with acetylacetone to form acetylacetone aluminum complex, while the amino end of protein can react with acetylacetone and formaldehyde to produce dihydropyridine products. The above compounds are absorbed at the wavelength of visible light, which affects the determination of samples. Therefore, the sample is prepared and loaded after centrifugation.

3.3. Selection of concentration of recovery test solution

The accuracy requirement in general rule 9101 (guiding principles for the validation of analytical methods) of Chinese Pharmacopoeia 2020 is to take the test sample of the same concentration within the specified range and evaluate it with the determination results of at least 6 samples; or design at least 3 different concentrations, and evaluate the determination results of at least 9 samples, and the concentration setting should consider the concentration range of the sample[7]. Recombinant hepatitis B vaccine (Saccharomyces cerevisiae). Adsorbed acellular DTP combined vaccine. Residues of formaldehyde in samples of split influenza vaccine and inactivated hepatitis A vaccine (human diploid cells) were 1.3, 26, 32 and 0.08 μg·mL^{-1}, respectively. The formaldehyde concentration of the four samples varies greatly, and the matrix has certain differences, so each variety has been tested with different concentrations of spiked recovery. Because the

formaldehyde residue in the samples of inactivated hepatitis A vaccine (human diploid cells) is low, when the recovery test adopts the method of three different concentrations, the recovery measured value of the low concentration is close to the quantitative limit, and the result has a certain error, so the determination method of at least six samples of the same concentration is used for accuracy evaluation.

3.4. Comparison with the determination results of general rule 3207 of Chinese Pharmacopoeia 2020

According to the third part of Chinese Pharmacopoeia 2020 edition, fuchsin sulphurous acid colorimetry is used to determine the adsorbed acellular DPT combined vaccine. Free formaldehyde residue of influenza virus split vaccine. The concentration range of standard solution in magenta sulphurous acid colorimetry is 25~100 μg·mL^{-1}, if the formaldehyde residue in the sample is lower than the minimum concentration of the standard curve, the content cannot be accurately determined. Precolumn derivatization liquid chromatography was used respectively. The residue of free formaldehyde in the above two vaccines was determined by post column derivatization liquid chromatography and magenta sulphurous acid colorimetry. The results of some samples were different, which may be due to the low residue of free formaldehyde in the samples. The results are shown in **Table 10**.

Acetylacetone colorimetry was used to determine the residue of free formaldehyde in recombinant hepatitis B vaccine (Saccharomyces cerevisiae) and inactivated hepatitis A vaccine (human diploid cells). The concentration of standard solution of this method was 0.25~100 μg·mL^{-1}. It is found that when the concentration of standard solution is 1.0 μ. When G·mL^{-1}, the absorbance is less than 0.03; when the concentration of standard solution is 50 μ. When G·mL^{-1}, the absorbance is greater than 1.0, which is beyond the recommended range of absorbance reading between 0.3 and 0.7 in the general rule 0401 (ultraviolet visible spectrophotometry) of Chinese Pharmacopoeia 2020 edition[7]. Precolumn derivatization liquid chromatography was used respectively. The residue of free formaldehyde in the above two vaccines was determined by post column derivatization liquid chromatography and fuchsin sulphurous acid colorimetry, and the results were relatively consistent.

4. Summary

In this study, pre column derivatization liquid chromatography and post column derivatization liquid chromatography were used to determine the residue of free formaldehyde in the vaccine. The results showed that the two methods could accurately determine the residue of free formaldehyde, and there was no significant difference in the determination results. Compared with fuchsin sulphurous acid colorimetry and acetylacetone colorimetry, pre column derivatization liquid chromatography and post column derivatization liquid chromatography are more specific, more sensitive and accurate. They can be used as quality control methods for free formaldehyde residues in vaccines.

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Conflict of interest

The authors declare no conflict of interest.

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