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Establishment and Verification of a Method for Detecting Trypsin Residues

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Abstract: Establish a detection method for residual trypsin and validate the method. Using enzyme-linked immunosorbent assay (ELISA) to detect residual trypsin in the single virus harvest solution and stock solution of oral trivalent reconstituted rotavirus attenuated live vaccine (Vero cells) (hereinafter referred to as trivalent rotavirus vaccine). At the same time, according to the requirements of Part 9101 of the Chinese Pharmacopoeia (2020 edition), the specificity, accuracy, repeatability, intermediate precision, linearity, durability, quantification limit and other indicators of the method are evaluated. The specificity verification results show that the recovery rates of recombinant trypsin standards at each concentration level are within the range of 70% to 125%, and the impact of MEM on the recovery rates of standards can be ignored. The accuracy verification results show that the recovery rates of the three concentrations of high, medium, and low (5ng/mL, 2.5ng/mL, 1.25ng/mL) diluted standard samples are all between 70% and 125%. The repeatability verification results showed that the relative standard deviation (RSD) of the six measurements of the trivalent rotavirus vaccine stock solution and the single virus harvest solution were both less than 15%. The intermediate precision validation results showed that the relative standard deviation (RSD) of the six measurements conducted by the two researchers on the trivalent rotavirus vaccine stock solution and the single virus harvest solution were both less than 15%. The standard curve plotted by the Logistic five parameter fitting curve calculation method based on the results of three freeze-drying standard sample tests for linear validation has a correlation coefficient R2 greater than 0.98. The durability validation results showed that there was no significant difference ($P>0.05$) between the three enzyme-linked immunosorbent assay (ELISA) results under 36℃ and 38℃ conditions and those under 37℃ conditions. The quantitative limit validation results show that after diluting the freeze-dried standard to the theoretical minimum value, the bottom limit value that is closest to the theoretical value and has the highest recovery rate is 0.312ng/mL. The enzyme-linked immunosorbent assay (ELISA) is suitable for the determination of residual trypsin in the single virus harvest solution and stock solution of trivalent rotavirus vaccines.

Keywords: Trypsin; Residual Amount; Enzyme Linked Immunosorbent Assay; Rotavirus; Validation of Analytical Methods.

1. Introduction

Rotavirus is the main cause of severe dehydrated gastroenteritis in children under 5 years old worldwide[1, 2]. Currently, there is no specific antiviral treatment for rotavirus. Once severe symptoms occur, the only clinically effective intervention is fluid replacement therapy. In emergency rooms and children who require hospitalization, the characteristics of rotavirus disease are usually watery diarrhea, vomiting, and fever, which can lead to electrolyte imbalance, shock, and in some cases even death[3]. At present, vaccination against rotavirus is the most effective method for preventing and controlling rotavirus infection. Vaccination against rotavirus has led to a significant reduction in diarrhea deaths and hospitalizations[1, 4].

Based on the natural reassortment characteristics of rotavirus [5], Lanzhou Institute of Biological Products Co., Ltd. (hereinafter referred to as Lanzhou Company) inserted the human rotavirus VP7 gene into the already marketed LLR strain rotavirus vaccine and cultured it on Vero cells to produce a reassorted G2, G3, and G4 trivalent rotavirus vaccine to prevent rotavirus induced diarrhea. Unlike other mammalian reoviruses, rotavirus requires a process of trypsin activation during cell culture. The VP4 protein, which forms the spike of the rotavirus shell, is a viral protein that binds to host cell receptors and directly determines the virulence of the virus. VP4 is a trypsin sensitive protein that is hydrolyzed by protease to form VP5 and VP8, which can enhance the ability of rotavirus to enter cells and enhance its infectivity[6, 7]. Trivalent rotavirus vaccine is an oral vaccine, which is used in a small amount in production(μg/mL) trypsin, whether from the perspective of injection vaccine or food safety, the residual amount of trypsin is very safe. However, since the trivalent rotavirus vaccine mainly serves infants, due to careful consideration of vaccine safety, we hereby carry out quality control on the residual amount caused by trypsin added in the critical stage of trivalent rotavirus vaccine production. Impurities and residues during vaccine production can also be used to verify the consistency of production process, such as residual host protein, residual cell DNA, endotoxin, bovine serum, trypsin and antibiotics [8]. Therefore, we need to establish a method to detect trypsin residues, whether from the perspective of the safety of the rotavirus vaccine or the requirements of the consistency of the rotavirus vaccine production process.

Enzyme linked immunosorbent assay (ELISA) is a method that combines the specific reaction of antigen antibody on the solid carrier with the enzyme catalytic substrate to quantitatively analyze the substance to be tested in the test sample. In this paper, it is proposed to detect trypsin residues in samples by ELISA method, and verify the specificity, accuracy, repeatability, intermediate precision, linearity, durability and quantitative limit of this method according to the requirements of 9101, Part III of the Chinese Pharmacopoeia (2020 Edition). The trivalent rotavirus vaccine includes LD_9(G2), $LS_{4/9}$(G3), and LH_9(G4), which

correspond to three types of single virus harvesting solution and three types of stock solution. During the inoculation and cultivation of each type of virus, the requirements for the use of trypsin are completely consistent. Therefore, this paper selects one type of single virus harvesting solution and stock solution as the validation material to confirm that this method is applicable to the determination of residual trypsin in the trivalent rotavirus vaccine of Lanzhou Company.

2. Materials and Methods

2.1. Sample

The single virus harvest solution and stock solution of the trivalent rotavirus vaccine are produced by Lanzhou Company.

2.2. Main Reagents and Instruments

The recombinant trypsin enzyme-linked immunosorbent assay kit was purchased from Shanghai Yaxin Biotechnology Co., Ltd., with a capacity of 96T and containing freeze-dried standards and concentrated diluents. The 1555 constant temperature incubator was purchased from SHEL LAB in the United States, and the Perkinelmer EnSight multifunctional ELISA reader was purchased from Perkinelmer Instruments Co., Ltd. MEM culture medium, purchased from BBI Solutions in the UK; Purified water is prepared by Lanzhou Company.

2.3. Preparation of the Solution

2.3.1. Diluent Preparation

Dilute the concentrated standard product & Sample diluent & HRP-antibody diluent with purified water at a volume ratio of 1:24.

2.3.2. Preparation of Washing Solution

Dilute the concentrated washing solution with purified water at 1:24 volume ratio.

2.3.3. Preparation of HRP Labeled Antibody Working Solution

Dilute the HRP labeled antibody with a dilution solution prepared in 1.3.1 in a volume ratio of 1:250.

2.3.4. Preparation of Working Solution for Color Substrate (TMB)

Dilute the TMB chromogenic substrate with substrate diluent in a volume ratio of 1:20.

2.3.5. Preparation of Standard Working Solution

Add 1.0mL of diluent to the freeze-dried standard, fully dissolve and mix to a concentration of 10ng/mL, and then dilute in multiples to obtain working concentrations of 10ng/mL, 5, 2.5, 1.25, 0.63, 0.312, 0.156, and 0ng/mL.1.4 Establishment of the method.

2.4. Establishment of Methods

2.4.1. ELISA Detection

Add the standard working solution to the first two rows of wells in sequence, and add two wells in parallel for each concentration of working solution, with a volume of 100 μL per well. Add the sample to other wells, 100 μL/well, and make two wells for each dilution. Dilution solution is directly used as a blank well, are well, and 100 μL/well. Cover the enzyme-linked immunosorbent assay (ELISA) plate with film and incubate at 37℃ for 60 minutes. Shake off the liquid in the hole, pat dry on a clean and paper free absorbent paper or dry cloth, add 300 μ L of washing solution to each hole, soak for 2-3 minutes, shake off the liquid in the hole, pat dry on a clean and paper free absorbent paper or dry cloth, and repeat this washing step 3 times. Add HRP labeled antibody working solution to the enzyme-linked immunosorbent assay (ELISA) plate, with a volume of 100 μL/well. Gently shake and mix well. After coating, incubate at 37℃ for 30 minutes. Wash the plate 4 times using the previous washing steps. Add 100 μL/well of TMB working solution to the enzyme-linked immunosorbent assay (ELISA) plate, cover with film, and incubate at 37℃ for 10 minutes. Add a termination solution of 50 μL/well to the enzyme-linked immunosorbent assay (ELISA) plate, terminate the reaction, and let it stand at room temperature for 1 minute. Read the absorbance values of each well at 450nm and 650nm on the ELISA reader.

2.4.2. Result Analysis and Calculation

Calculate the average OD value for each group of composite holes. The average OD value of each standard is subtracted from the OD value of the blank hole as the correction value. Draw the standard curve using the Logistic five parameter fitting curve calculation method, with the concentration of the standard substance as the x-axis (X) and the OD value as the y-axis (Y). The OD values of the test sample are within the curve range of the standard sample (10, 5, 2.5, 1.25, 0.63, 0.312, 0.156, 0 ng/mL), and the results are reliable.

2.5. Verification of the Method

2.5.1. Accuracy

Confirm that the method's measured value is within an acceptable range of approximation to the theoretical value. Experimental design: The standard working liquid of high, medium and low concentrations (5 ng/mL, 2.5 ng/mL, 1.25 ng/mL) was prepared according to 1.3.5, and the test was carried out according to the method established in 1.4. Each concentration was measured three times, and the measured concentration value was compared with the theoretical concentration value. The approximation between the two was expressed by the recovery rate (Rx). To calculate whether the recovery rate is within the acceptable standard 70%-125%.

Recovery Rx=C1/C0×100% (C1 is the determination concentration value of high, medium and low concentration samples; C0 is the theoretical concentration value of high, medium and low concentration samples.)

2.5.2. Specificity

Confirm that the accuracy of this method is not disturbed when other components (MEM) are present in the sample. Experimental design: Dilute the freeze-dried standard with MEM culture medium instead of the diluent in the method, and examine whether the accuracy of the method is affected (the recovery rate calculation method is the same as specified in 1.5.1).

2.5.3. Repeatability

The same experimenter conducted 6 measurements on the same batch of trivalent rotavirus vaccine stock solution and single virus harvest solution using the method established in 1.4, and calculated the degree of closeness between the test results (represented by relative standard deviation RSD). If the RSD between the detection results of the original solution and the single virus harvest solution is ≤ 15%, it indicates that the method has repeatability.

2.5.4. Intermediate Precision

Two testers measured the same batch of trivalent rotavirus vaccine stock solution and single virus harvest solution for six

times respectively according to the method established in 1.4, and calculated the closeness between the six test results of the same sample by two testers (expressed by relative standard deviation RSD). If the RSD of the two groups is ≤ 15%, the intermediate precision of the method is good.

2.5.5. Linearity

Confirm the ability that the OD value result of the linear test of the standard used in this method is directly proportional to the concentration. Experimental design: According to the method established in 1.4, the OD values of 7 linear concentration levels (10, 5, 2.5, 1.25, 0.63, 0.312, 0.156 ng/mL) of the standard were detected. Calculate the average OD value of each standard, and subtract the OD value of the blank hole from each average OD value as the correction value. With the concentration of the standard as the abscissa (X) and the OD value as the ordinate (Y), use the Logistic five parameter fitting curve calculation method to draw the standard curve, obtain the correlation coefficient R^2, and determine whether the method is linear. The test was repeated three times.

2.5.6. Durability

Confirm the tolerance that the test results of this method will not be affected when there is a small change in the test conditions. Experimental design: The same batch of trivalent rotavirus vaccine stock solution and single virus harvest solution were added according to the sampling rules in the method established in 1.4, and the enzyme labeling test was carried out at 36℃ and 38℃ respectively. Each sample to be tested was repeated 8 times at the same temperature. Finally, the test results at two temperatures were paired with the test results at 37℃ for nonparametric test to see whether there was a significant difference (P>0.05, no significant difference).

2.5.7. Limit of Quantification

Investigate the lowest amount of the tested object in the sample that can always be measured quantitatively. Experimental design: Dilute 10ng/mL standard solution with diluent, and take 1.25, 0.625, 0.312, 0.156, 0.078, 0.039, 0.0195, 0.00975, 0.004875, 0.0024375, 0.00121875 and 0.000609375 ng/mL standard diluent as the sample to be tested. Repeat the test for three times according to the method established in 1.4, and take the lowest concentration value that can be quantitatively determined in three tests and meets the accuracy requirements of the method as the quantitative limit of the method.

3. Results

3.1. Accuracy

The results show that the recoveries of standard diluents of high, medium and low concentrations (5 ng/mL, 2.5 ng/mL, 1.25 ng/mL) are between 70% and 125%, as shown in Table 1.

Table 1. Recovery rates of diluents of three concentration standard substances

Theoretical concentration value C0 (ng/mL)	Measured concentration value C1 (ng/mL)	Recovery Rx (%)
5	3.81764	76.35
	3.71521	74.30
	3.54297	70.86
2.5	2.09385	83.75
	1.94965	77.99
	2.06259	82.50
1.25	1.02018	81.61
	0.93835	75.07
	1.02793	82.23

3.2. Specificity

The results show that the recoveries of standards at each concentration level are in the range of 70%~125%, as shown in Table 2. The influence of MEM on the recovery rate of standard can be ignored. When there are other components (MEM) in the sample, the accuracy of the method will not be disturbed.

Table 2. Recovery rates of diluents of three concentration standards when MEM is present

Theoretical concentration value C0 (ng/mL)	Measured concentration value C1 (ng/mL)	Recovery rate Rx (%)
5	4.67331	93.47
	6.01229	120.25
	3.87579	77.52
2.5	2.49839	99.94
	3.00760	120.30
	2.02461	80.98
1.25	1.13699	90.96
	1.44506	115.60
	1.14401	91.52

3.3. Repeatability

The results showed that the relative standard deviation (RSD) of six determinations of trivalent rotavirus vaccine stock solution and single virus harvest solution were 10.84% and 8.44% respectively, as shown in Table 3.

Table 3. Relative standard deviation (RSD) of 6 tests results of trivalent Rotavirus vaccine stock solution and single virus harvest solution

Samples	1	2	3	4	5	6	$\bar{x}$ (ng/mL)	RSD (%)
Stock solution	7.18	5.45	5.74	5.47	5.83	6.14	5.93	10.84
Single virus harvest solution	3.45	3.79	3.47	3.14	3.15	3.03	3.34	8.44

3.4. Intermediate Precision

The results showed that the relative standard deviations of the six determinations of the trivalent rotavirus vaccine stock solution and the single virus harvest solution by the two testers were 8.18% and 7.03%, respectively, as shown in Table 4.

Table 4. The relative standard deviations of 6 determination results of trivalent Rotavirus vaccine stock solution and single virus harvest solution

Samples	1	2	3	4	5	6	$\bar{x}$ (ng/mL)	RSD (%)
Stock solution a	4.12	3.69	3.93	4.66	4.09	4.48	4.15	8.18
Stock solution b	3.70	4.44	4.31	3.85	4.58	3.93		
Single virus harvest solution a	2.34	2.45	2.59	2.24	2.26	2.13	2.40	7.03
Single virus harvest solution b	2.33	2.55	2.68	2.56	2.40	2.27		

3.5. Linearity

Table 5. Correlation coefficient of the standard curve of the three freeze-dried standard products testing

Number of Tests	1	2	3
Correlation coefficient (R^2)	0.9999	0.9997	0.9999

The results show that the correlation coefficient R^2 of the test standard curve of the three freeze-dried standards is greater than 0.98, as shown in Table 5.

3.6. Durability

The nonparametric test results show that there is no significant difference (P>0.05) between the results of three enzyme labeling tests at 36 ℃ and 38 ℃ and those at 37 ℃, as shown in Table 6.

Table 6. Comparison of enzyme-labeled test results under three temperature conditions

Times	Stock solution test results (ng/mL)			Single virus harvest liquid detection results(ng/mL)		
	36℃	37℃	38℃	36℃	37℃	38℃
1	5.40983	5.44244	6.95988	2.39178	2.8441	3.15794
2	5.22338	5.54558	5.15513	1.88407	1.83398	2.10523
3	5.65287	6.11553	6.27888	1.90645	2.45724	2.73712
4	6.70279	6.43886	5.9179	2.22286	2.37711	2.60045
5	6.39517	5.91751	7.59641	2.17885	2.6049	2.7119
6	6.70681	5.04235	7.15049	2.24472	2.5819	2.56053
7	6.71691	5.32146	7.75779	3.90786	2.66838	2.76061
8	9.38234	5.39185	10.0446	3.38207	3.72481	3.45156
Nonparametric test $P_{36/37}$	0.19		/	0.57		/
Nonparametric test $P_{38/37}$	/	0.16		/	0.12	

3.7. Limit of Quantitation

The results show that after the lyophilized standard is diluted to the theoretical minimum value, the bottom limit value of the measured value and the theoretical value are the closest and the highest recovery rate is 0.312 ng/mL, as shown in Table 7.

Table 7. Comparison between the measured value and the theoretical value

Theoretical Values (ng/mL)	Measured value (ng/mL)		
/	1	2	3
1.25	1.25703	1.18094	1.09456
0.625	0.579984	0.494089	0.555622
0.312	0.229708	0.229707	0.250043
0.156	0.0877476	0.0675056	0.112956
0.078	0.0175760	0.0141773	0.0476537
0.039	/	/	/

4. Discuss

In this method, the standard curve is obtained by subtracting the OD value of the blank hole from the average OD value of each standard as the correction value, taking the concentration of the standard as the abscissa (X) and the OD value as the ordinate (Y), and using the Logistic five parameter fitting curve calculation method. The sample OD value is within the range of the standard curve, and the result is credible. If the sample OD value is higher than the upper limit of the standard curve, it should be properly diluted and retested. In the production process of trivalent rotavirus vaccine, the initial concentration of trypsin is 1 μg/mL. Before the start of this validation, we conducted a preliminary experiment on trypsin residues in the samples, accumulated a certain amount of data, and found that the OD values of trypsin residues in the relevant process stages of the trivalent rotavirus vaccine were within the range of the standard curve used in this method. Therefore, in the detection of trypsin residues in the single virus harvest solution and stock solution of the trivalent reconstituted rotavirus vaccine, it was not necessary to dilute the samples again to ensure the credibility of the detection results. All working solutions involved in this method shall be prepared according to the principle of ready to use, and the TMB working solution shall be placed away from light after preparation. The recombinant trypsin has the same enzymatic activity and specificity as the natural trypsin,

so the recombinant trypsin detection kit has great reference value for the detection of natural trypsin.

The specificity, accuracy, repeatability, intermediate precision, linearity, durability, quantitative limit and other indicators of the established ELISA method for trypsin residue detection were verified according to the requirements of 9101 Guiding Principles for Validation of Analytical Methods, Part III of the Chinese Pharmacopoeia (2020 Edition). The verification text has been approved after inspection and confirmation before the implementation of verification. All documents, equipment, instruments and meters, materials and reagents, verification executors and other items needed to be confirmed during the verification process meet the acceptable standards. According to the requirements of the predetermined validation scheme and relevant reference documents, all validation projects have been completed and meet the acceptable standards. This method can be used for the detection of trypsin residues in trivalent rotavirus vaccine. The actual detection should be carried out in strict accordance with the conditions and operating procedures that have been determined by this method. Unverified methods and operating procedures should not be used for the determination. When the test article or method changes, the method needs to be revalidated[5-7, 9-12].

Vaccine is a special drug. Its particularity lies in that it is used for healthy people to prevent infectious diseases, most of which are used for infants and newborns. Vaccine safety is the primary task in vaccine quality control. Trypsin is required to participate in the process of rotavirus infection of cells. Trypsin residue detection is required in the subsequent production part of the process. At the same time, impurities and residues (such as trypsin) in the production process can be used to verify the consistency of the production process. This is also our original intention to establish an enzyme-linked immunosorbent assay for the detection of trypsin residues[8].

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