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Development and Validation of HPLC Method for Analysis of Impurities of Fosfluconazole in Pharmaceutical Products

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Authors' contributions:

This work was carried out in collaboration between all authors. Author LL designed the study and wrote the protocol. Authors YH and XY managed the literature searches and analyses of the study performed the spectroscopy analysis. Author QL managed the experimental process, preformed the statistical analysis and wrote the first draft of the manuscript with assistance from authors JL and JC. All authors read and approved the final manuscript.

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ABSTRACT

The contents of three related substances in fosfluconazole were determined by high performance liquid chromatography (HPLC). The limit of detection (LOD) of the impurity A (2-(2,4-difluorophenyl)-1,3-bis (1H-1,2,4-triazole-1-yl)-2-propan-2-ol), the impurity B (2-(2,4-Difluorophenyl) -1-(1H-1,2,4-triazol-1-yl) -3-(4H-1,2,4- triazol-4-yl) propan-2-yl dihydrogen phosphate) and the impurity C (2-(2-fluorophenyl) -1,3-bis (1H-1,2,4-triazole -1-yl)-2-propyl dihydrogen phosphate) were 3.0, 3.0 and 5.0 μg/mL respectively. Limit of quantification (LOQ) were 10.0, 22.0 and 21.3 μg/mL for A, B and C. The method can be used for the quality control of the related substances in fluconazole injection, tablet and capsule.

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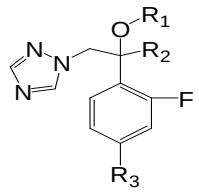
Keywords: Fosfluconazole; fluconazol; impurity; HPLC.

1. INTRODUCTION

Fosfluconazole, the trade name of Prodif, which chemical name is 2-(2,4 -Difluorophenyl)-1,3-bis(1H-1,2,4-triazole-1-yl)-2-propyl dihydrogen phosphate [1]. Fosfluconazole is phosphate ester prodrug of fluconazole and it is a new antifungal drugs of triazoles. It was alkaline phosphatase hydrolyzed to fluconazole and acid in the body in order to achieve the effect of treatment of fungal infection [2]. Fosfluconazole is developed by the Central Research Institute of Pfizer Inc of the United States on October 16, 2003 in Japan and using to the treat fungal disease caused by candida, cryptococcus; respiratory, digestive tract, urinary tract of fungal disease, peritonitis, meningitis, etc [3-4]. It is very important to improve medication safety reliability of fosfluconazole, achieve the purpose of effective control of the quality and study on the impurities in pharmaceutical products. Therefore, it is necessary to control the impurity in a safe and reasonable range in the new drug [5].

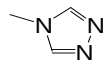
The main impurity structure of fosfluconazole in the synthesis process has shown in Fig.1, 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazole-1-yl)-2-propan -2-ol (impurity A), 2-(2,4-Difluorophenyl)-1-(1H-1,2,4-triazol-1-yl)-3-(4H-1,2,4 -triazol- 4-yl) propan-2-yl dihydrogen phosphate(impurity B), 2-(2-fluorophenyl)-1,3-bis(1H-1,2,4-triazole-1-yl)-2-propyl dihydrogen phosphate(impurity C) [6]. The impurities are mainly produced by synthesis, and one of the synthetic routes was shown in Fig. 2.

So far, fosfluconazole was not included in the Chinese Pharmacopoeia 2015 Edition (ChP 2015), the United States Pharmacopoeia 34 Edition (USP 34), the European Pharmacopoeia 6 edition (EP 6) and the Japanese medicine 15 Edition (JP 15). And the analysis method of fosfluconazole related substances is still relatively rare. Wang Y et. al. [7] screened and optimized the elution conditions of fosfluconazole related substances and the method showed good specificity and short analysis time. However, the related substances B and C cannot be separated well due to the low purity of the impurities and the analysis method. Thus, the more optimized method to detect the related substances of fosfluconazole is needed.



Fosfluconazole: $R_1=PO_3H_2$, $R_2=$  , $R_3=F$

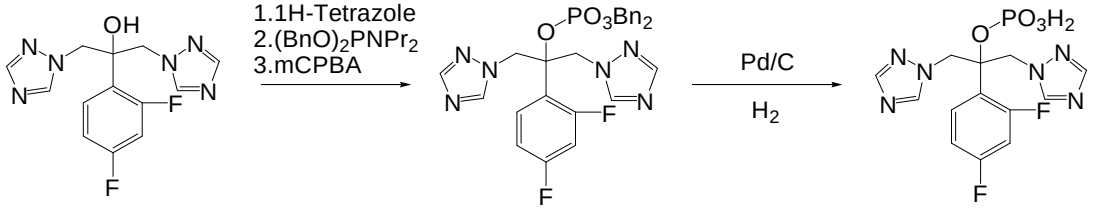
Impurity A : $R_1=OH$, $R_2=$  , $R_3=F$

Impurity B : $R_1=PO_3H_2$, $R_2=$  , $R_3=F$

Impurity C : $R_1=PO_3H_2$, $R_2=$  , $R_3=H$

Fig. 1. The chemical structure of fosfluconazole, impurities A, impurity B, and impurity C

In this paper, we reported the development and optimization of a method by HPLC analysis methods for determination of the three impurities. We have made a quantitative methodology study on impurity A, B and C, and have developed the corresponding control method [8], which would provide a theoretical basis for the quality control of the synthesis process of fosfluconazole and the construction of the quality standard of the finished product.



Fluconazole **Fosfluconazole**

Fig. 2. Synthesis route of fosfluconazole

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Standard of fosfluconazole (99.5% of purity), the impurity B(98.0% of purity) and the impurity C(97.9% of purity) were obtained from the School of Life Science and Engineering, Southwest University of Science and Technology. Standard of the impurity A(99.0% of purity) was purchased from Zhejiang Genebest Pharmaceutical Co., Ltd. Acetonitrile, methanol, of HPLC grade, were supplied by Thermo Fisher Scientific. Potassium phosphate monobasic was of analytical-reagent grade and supplied by Chengdu Kelong Reagent Co., Ltd. HPLC grade water was purified by redistillation and passed through a 0.45 μm Millipore filter before use.

2.2 Instrumentation

The chromatograph consisted of an Agilent 1260 HPLC system with G1311C quaternary pump, G1315D DAD array detector and variable wavelength detector, and a DT230A column temperature box. The data were evaluated by Chemstation B.04.03 chromatography workstation. The 5500PC ultraviolet visible spectrophotometer was used for spectral scanning. The Milli-Q ultrapure water machine was used for purified water.

2.3 Chromatographic Conditions

The method was developed using an Agilent Explise XDB-C18, 250 mm×4.6 mm, 5 μm column as the stationary phase. Mobile phase A consisted of 0.02 M phosphate buffer. Mobile phase B consisted of pure acetonitrile [9]. The gradient program time (minutes)/% mobile phase B (%B) was set as Table 1. The mobile phase was pumped at 0.8 mL/min. The fosfluconazole, impurity B and impurity C were monitored at 260 nm. The column temperature was maintained at 25℃. The injection volume for samples and standards was set at 5.0 μL. The number of the theoretical plate is not less than 3000, and the separation degree of the fosfluconazole and the related substances are all meet the requirements [10].

2.4 Preparation of Solutions

2.4.1 Preparation of standard solution

Standard stock solutions of drug substance was prepared by dissolving 25.0 mg of fosfluconazole, in 25 mL of HPLC-grade solvent (methanol:water=1:2, v/v) in 25 mL volumetric flask to obtain a concentration of 1.0 mg/mL of fosfluconazole. The same method is used for the impurity A, B and C. Then sonicated for 20 minutes, allowed the sample to attain room temperature and made up the volume to the mark with water. The standard solutions were filtered with 0.45 μm Nylon filter (Millipore, USA) by discarding first few mL of the solution.

Table 1. The procedure of gradient elution

Time/min	Gradient elution(A: B /v:v)
0~8	90：10
8~15	90：10→80：20
15~30	80：20→60：40

2.4.2 Preparation of resolution solution

The resolution of drug were prepared by dissolving 25.0 mg of impurities and diluted to 25 mL with HPLC-grade solvent(methanol:water=1:2, v/v) to get resolution solution as a concentration of 1.0 mg/mL. Then sonicated for 20 minutes, allowed the sample to attain room temperature and made up the volume to the mark with water. The resolution solution was filtered with 0.45 μm Nylon filter (Millipore, USA) by discarding first few mL of the solution.

2.4.3 Preparation of sample solution

Weighted a batch of sample of fosfluconazole to 25.0 mg and dissolved the sample by 25 mL with HPLC-grade solvent (methanol:water=1:2, v/v) in 25 mL volumetric flask to get sample solution as a concentration of 1.0 mg/mL. Then sonicated for 20 minutes, allowed the sample to attain room temperature and made up the volume to the mark with water. The sample solution was filtered with 0.45 μm Nylon filter (Millipore, USA) by discarding first few mL of the solution.

2.5 Method Validation

This method was validated by linearity, limits of detection and quantification, precision and accuracy, stability in solution, recovery, repetitive experiments, robustness and determination of relevant substances.

2.5.1 Linearity

The linearity study is carried out from a series of working resolution solutions of 1, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, 0.025, 0.01, 0.005 mg/mL of

impurity A, impurity B, impurity C and fosfluconazole. Standard plots were constructed and linearity was evaluated statistically by linear regression analysis that was calculated by least-squares regression.

2.5.2 Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) for impurities were established at S/N (Signal to Noise) = 3 and S/N = 10 and determined based on the residual standard deviation of a regression line and slope method by injecting a series of dilute solutions with known concentrations.

2.5.3 Precision

The precision of the proposed method was evaluated by six replicate injections of fosfluconazole standard solutions which were given into the HPLC system to establish system precision. The instrument precision was evaluated by injecting the same solution for six times and calculated by %RSD. The fosfluconazole standard solution was analyzed on the same day in precision study.

2.5.4 Stability in solution

The stability of the fosfluconazole standard solutions was tested by injecting the same test solution at 0 h, 2 h, 4 h, 6 h, 8 h and 24 h. Then, the RSD (%) of the assay of fosfluconazole was calculated.

2.5.5 Repetitive experiments

The repetitive of the fosfluconazole standard solutions was evaluated by injecting the same solution into the HPLC system for six times and calculated by %RSD.

2.5.6 Recovery

Added impurity A, impurity B and impurity C into fosfluconazole standard solutions to prepared a new sample. And two samples with mass of 400 and 665 ng were prepared in triplicate and analyzed by developed HPLC method. Then, the ratio of the recorded peak heights to the peak heights resulted from the direct injection of the aqueous solutions of toltrodine with the same mass were determined as percentage in each case. The injection volume for samples was set at 5.0 µL.

2.5.7 Robustness

To establish the robustness of the method, experimental conditions were deliberately changed, and the resolution between these impurities was evaluated. Such as the acetonitrile concentration, the pH value and so on. The degree of reproducibility was evaluated by the resolution between fosfluconazole and its impurities.

2.5.8 Determination of relevant substances

The determination of relevant substances of the fosfluconazole sample solutions was tested by injecting the same test solution for three times. Then, the content (%) of impurity A, B and C were calculated.

3. RESULTS AND DISCUSSION

3.1 Method Development

3.1.1 The selection of detection wavelength

To obtain the appropriate wavelength for determination of fosfluconazole and the impurities, solutions of these compounds in the mobile phase were scanned by UV–visible spectrophotometry in the range 190–400 nm [11]. From the scanned results (Fig. 3), the suitable wavelength choices for monitoring impurities A, B and C were 210 nm and 260 nm. Contrast with directly determination test of 210 nm for HPLC analysis, no interference from the mobile phase or baseline disturbance showed at 260 nm. Thus, the most appropriate wavelength for analysis of the four substances with suitable sensitivity was chosen as 260 nm.

3.1.2 The selection of mobile phase and analysis method

A gradient HPLC method was adopted to get a shorter runtime and higher sensitivity after several tests. Because the four substances had triazol group, the pH of mobile phase would affect the peak shape and resolution. The results showed that the pH of mobile phases was important that the retention times of impurity A was lengthened when the amount of phosphate was increased. The various gradient programs were also tried to get better resolution and shorter separation time. After hard work, suitable pH and ratios of mobile phases and the gradient program (acetonitrile and 20 mM potassium dihydrogen phosphate aqueous solution, pH=5.0, Table 1) were found.

Dongre VG et al. [8] proposed a convenient HPLC method (with the mobile phase of water and acetonitrile in the ratio of 80:20) for the detection of impurities in fluconazole. We improved the method because of the differences of functional groups between fosfluconazole and fluconazole. Compared to the Wang Y [7] et al. it showed good specificity and short analysis time, but the related substances B and C cannot be separated well. So our method was given shorter running time, better resolution and good symmetric peaks (Fig. 4).

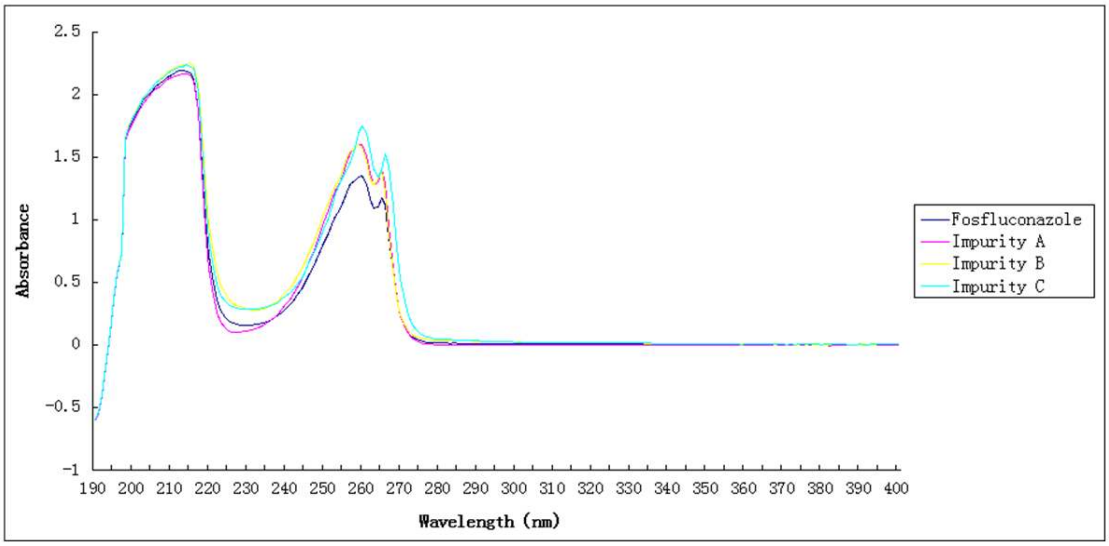
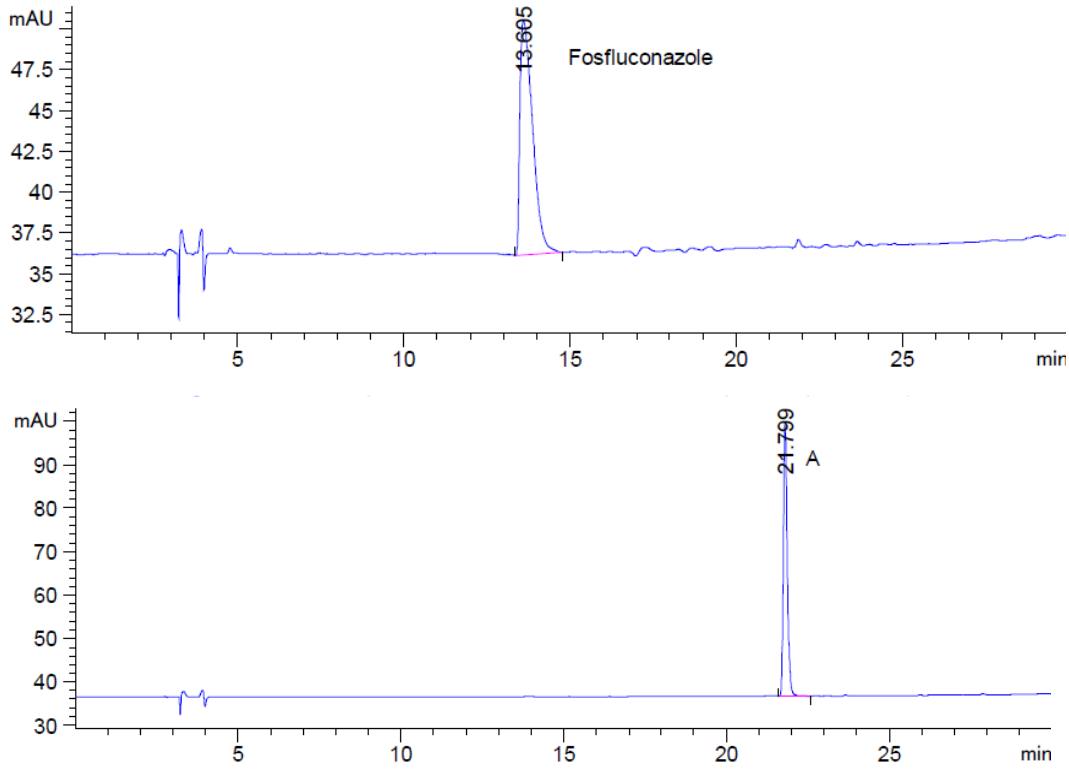


Fig. 3. The UV spectra of fosfluconazole, impurity A, B and C



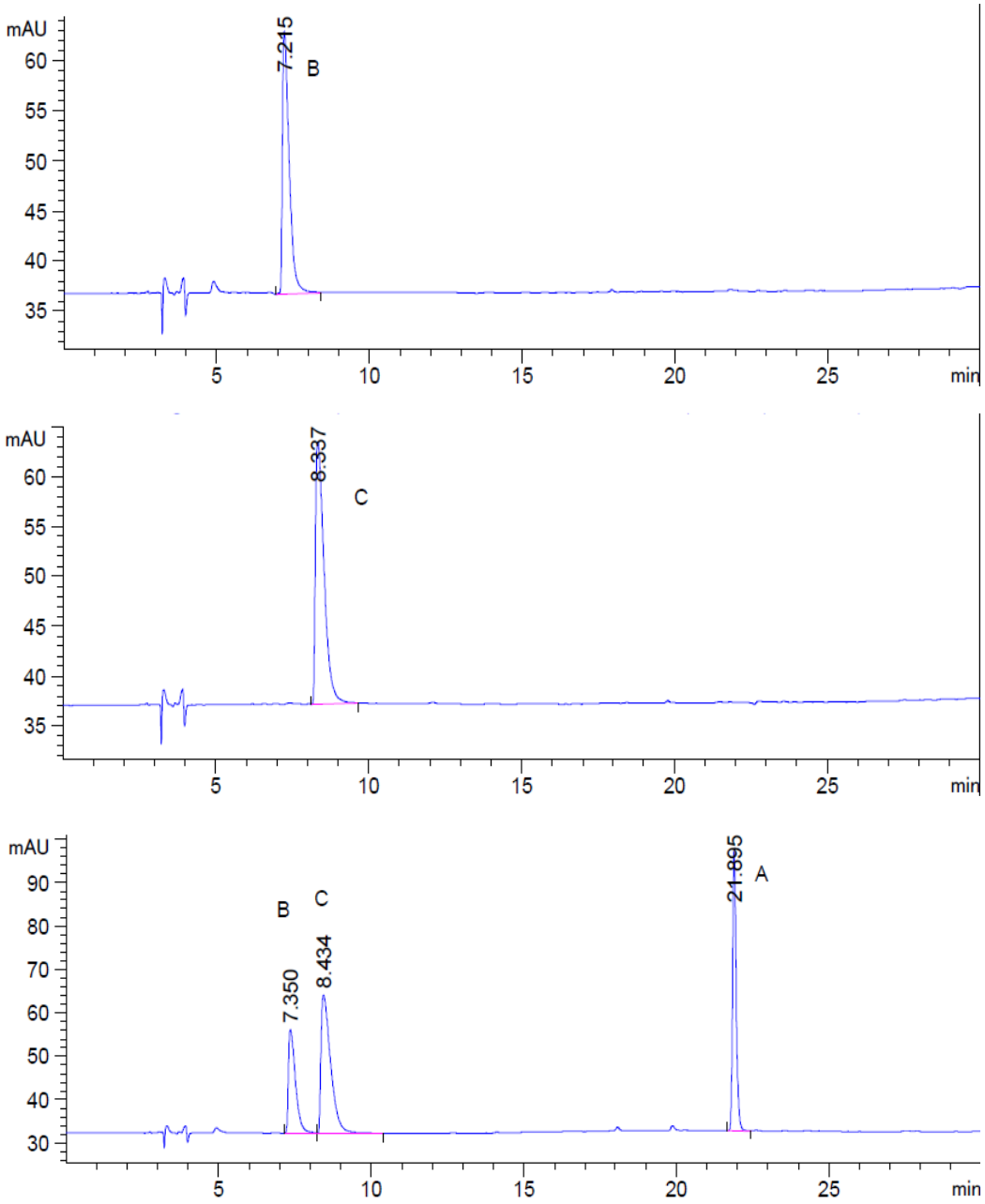


Fig. 4. The HPLC chromatograms of fosfluconazole, impurity A, impurity B , impurity C and their mixture (from up to down)

3.2 Method Validation

3.2.1 Linearity

Linearity of the proposed HPLC procedure was evaluated by analyzing a series of different concentrations for each of the three impurities. According to the HPLC spectrum, linear regression equations were generated by least squares treatment of the calibration data and the concentration (X) was set as the horizontal coordinates and the peak area (Y) for the vertical coordinate, R^2 showed the square of determined correlation coefficient. The linear regression equation was obtained as Y = 468.20X+6.6093 (R^2 = 0.9966) for impurity A, Y = 335.63X+2.5958(R^2 = 0.9955) for impurity B, Y = 611.26X+3.4844(R^2 = 0.9956) for impurity C and

Y = 351.48X+11.8000(R^2 = 0.9995) for fosfluconazole. The results indicated that the method was linear over the concentration range 0.005–0.8 mg/mL due to the three R^2 values >0.99.

3.2.2 Limits of detection and quantification

Limits of detection (LOD) and quantification (LOQ), as a measure of method sensitivity, were provided for degradation products and impurity calculated by means of signal-to-noise ratio. The LOD and LOQ for impurity A, B and C are tabulated in Table 2. From the results, it can be concluded that the proposed method can quantify small quantity of impurities in Fosfluconazole samples.

3.2.3 Precision

The RSD of the proposed method precision with the fosfluconazole standard solution for six replicate injections for analyte was 1.3%, showing the sample repeatability and the system precision of the method were good.

3.2.4 Stability in solution

According to the results of the stability test of fosfluconazole standard solutions at 0 h, 2 h, 4 h, 6 h, 8 h and 24 h, the RSD (%) of the fosfluconazole peak area was 1.8%. The results showed that the stability of the fosfluconazole at room temperature for 24 h was good.

3.2.5 Repetitive experiments

According to the results, the RSD of the repetitive of the fosfluconazole peak time was 0.5%. The experimental results show that the method has good repeatability.

3.2.6 Recovery

The accuracy of the method was assessed by recovery test. Calculated recoveries and results are shown in Table 3. According to the results, the average recoveries of impurity A, B and C were all in 99.9% ~ 100.2% and all of the RSD < 0.5%. The experiments proved that the method is feasible.

3.2.7 Robustness

When the acetonitrile concentration and the pH value were changed, the resolution between fosfluconazole and the impurities was not significantly affected. The degree of change in the method parameters has proven that the method is robust.

3.2.8 Determination of relevant substances

According to the results of HPLC spectra and Fig. 3, the peak position of fosfluconazole and the impurity A, B and C were confirmed. Crude sample of synthesized fosfluconazole was analyzed using the developed method. The result of HPLC is shown as Fig. 5. And the content of fluconazole was 68.26%, the content of A was

Table 2. LOD and LOQ results for the three impurities

Compound	Injection volume (μL)	LOD		LOQ	
		Concentration (μg/mL)	Amount of detection(ng)	Concentration (μg/mL)	Amount of detection (ng)
Impurity A	5.0	3.0	15.0	10.0	50.0
Impurity B		5.0	25.0	22.0	110.0
Impurity C		5.0	25.0	21.3	106.3
Fosfluconazole		3.0	15.0	15.4	77.0

Table 3. Accuracy results [Recovery (%)] for the determination of impurities A, B and C

Compound	Added weight(ng)	Average recovery(%)	RSD(%)
Impurity A	400	100.1	0.28
	665	100.2	0.33
Impurity B	400	100.1	0.27
	665	99.9	0.48
Impurity C	400	100.2	0.31
	665	100.1	0.33
Fosfluconazole	4600	100.1	0.29
	4335	100.2	0.31

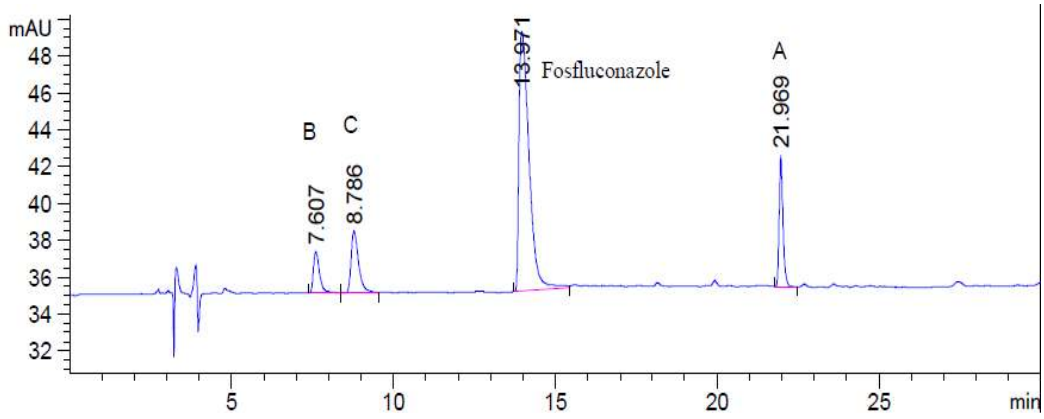


Fig. 5. HPLC chromatogram of the fosfluconazole crude sample

13.45%, the content of B was 6.57%, and the content of C was 11.72%. The results show that the method is feasible and can be used for the quantitative detection of fosfluconazole impurities.

4. CONCLUSION

The impurity A, B and C can be detected simultaneously in the experiment when the DAD detector is provided with 210 nm and 260 nm. The study proves that the impurity positioning method can be accurately carried out by using the known impurity control method, and the method has the advantages of strong specificity, high sensitivity, good precision, simple and rapid. It can check the content of the related substances in fosfluconazole effectively and provide some basis for the quality control of the synthesis process of the fosfluconazole and the construction of the quality standard of the finished product.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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