

# HPLC Method for the Determination Content of 5-HMF in Zishen Yutai Pill

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**Abstract:** Objective: During the preparation of Zishen Yutai Pills, 5-Hydroxymethylfurfural may be used. To establish a HPLC method for determination of 5-HMF in Zishen Yutai pill and to provide the potential quality control standard of it. Methods: RP-HPLC method was applied with the chromatographic condition as follows: the chromatographic column was Phenomenex Gemini C18 column (250×4.6mm, 5μm), methanol-water (10:90) as the mobile phase, the flow rate was 0.8mL/min, the detection wavelength was 284nm, the column temperature was 30°C, and the injection volume was 10μL. Results: The calibration curve was linear in the range of 2.16~43.27μg. mL<sup>-1</sup> for 5-HMF ( $r^2=0.9993$ ). The RSD of the method repeatability was 0.97% (n=6) and the average recovery was 99.4% (RSD=0.58%). What's more, the specificity, precision and stability results were also good. Conclusion: The method for HPLC is not only sensitive and accurate. It is also easy to operate which can be used as the determination method for 5-HMF and has a guiding role for the quality control of the Zishen Yutai pill.

**Keywords:** HPLC, Zishen Yutai Pill, 5-HMF, Determination, Methodology

## 1. Introduction

Zishen Yutai pill (ZYP) is a traditional Chinese medicine compound preparation containing 15 Chinese herbal medicines. ZYP has rich clinical experience in treating various diseases such as infertility and miscarriage [1]. Recently, it has been reported that it can enhance the receptivity of the endometrium, prevent re-abortion, and enhance the spleen and kidney function. Similarly, it is an effective medicine for the treatment of menstrual disorders with luteal phase deficiency, asthenospermia and spleen and kidney deficiency type infertility [2, 3].

The main ingredients of ZYP are Codonopsis, Polygonum multiflorum, Eucommia, Cuscuta, Rehmannia glutinosa, etc. According to the literature, the 5-HMF content of Rehmannia glutinosa after processing will increase about 20 times. Honey, as a binder for ZYP will cause monosaccharides such as fructose to be heated to remove tri-molecular water to form 5-HMF, which increases the content of monosaccharides such as fructose. There are related literature reports that the daily

allowable intake of 5-hydroxymethyl furfural is 30-60 mg, and foreign literature reports that the intake of 5-HMF is 1.6-150 mg/person/day. At present, there is a lot of controversy about 5-HMF discussion. According to reports in the literature, larger doses can damage human striated muscle and internal organs [4]. It is neurotoxic and can combine with human proteins to produce accumulated poisoning [5] and abnormal growth of colonic sac [6], which seriously affects the health of patients taking the drug. The "Chinese Pharmacopoeia" (2010 edition) [7] has clearly listed 5-HMF as an impurity item in the quality standards for glucose-containing injections for monitoring. The existing literature research focuses on the determination of 5-hydroxymethylfurfural content, and seldom involves the determination of 5-hydroxymethylfurfural in ZYP [8].

This article will focus on this issue. The establishment of a high performance liquid chromatography determination method aims to provide a reference for the quality control of the drug.

## 2. Methods

### 2.1. Instruments

Agilent 1260 high-performance liquid chromatograph, Agilent 1260 G4212B PDA detector, American Agilent company; Sartorius BT125D electronic balance, 1/100,000, Sartorius, Germany; Sartorius BSA124S electronic balance, 1/10,000, Sartorius, Germany Si company; H/T16MM desktop high-speed centrifuge, Hunan Hexi Instrument Equipment Co., Ltd.; KQ-300DE CNC ultrasonic cleaning instrument, Kunshan Ultrasonic Instrument Co., Ltd.; UPT-IV-20L ultrapure water system, Chengdu Yuyou Technology Co., Ltd.

### 2.2. Main Reagents

5-hydroxymethyl furfural reference substance was purchased from National institutes for food and drug control (Lot Number: 111626-201509) and ZYP was from Guangzhou Zhongyi Pharmaceutical Company (Lot Number: S00033, T00058, T00061, Guangzhou, China).

### 2.3. High-performance Liquid Chromatographic Analysis

Chromatographic column: Phenomenex Gemini C18 column (250mm×4.6mm, 5μm); mobile phase is methanol-water (10:90); volume flow rate is 0.8 mL/min; detection wavelength is 284nm; column temperature is 30°C; sample volume is 10μL.

### 2.4. Preparation of Standard Solutions

A standard stock solution of 5-HMF was prepared by dissolving 11.06 mg of 5-HMF reference substance, place it in a 10 mL brown measuring flask, add methanol to dissolve, dilute to the mark, and shake well. Precisely pipet 5 mL, place it in a 25 mL brown volumetric flask, add methanol to dilute to the mark, shake well, and get a mass concentration of 0.2212 mg·mL<sup>-1</sup> reference substance stock solution.

### 2.5. Preparation of Sample Solutions

The powder of ZYP samples quantitatively (0.2 g) transferred into round-bottomed flask and extracted with 25ml of mobile phase and weighed after well mixed, and then placed 12 h, Ultrasounded for 30 min (300W, 40kHz), and reweighed with deionized water added to make up the weight before centrifuged at 10000r·min for 30 min, then taken the supernatant as the test solution.

### 2.6. Preparation of Negative Blank Solution

The Chinese medicinal materials in relevant prescriptions were taken and the negative samples lacking 5-HMF were prepared according to the preparation process of ZYP sample, and the negative blank solution was prepared according to the item "2.5".

## 3. Results

### 3.1. Specificity Test

Under the chromatographic condition of "2.3", the reference solution, test solution and negative blank solution were sampled with 10μl each, and the chromatogram was recorded to observe whether other components interfered with the sample peak.

The results showed that at the corresponding position of 5-HMF chromatographic peak, the test product had the same retention peak with the retention time of about 10.96 min, while the negative sample solution had no absorption and no interference at this peak position.

### 3.2. Linear Relation Investigation

The reserve solution of reference was accurately removed into 10 mL volumetric flask 0.1, 0.3, 0.5, 0.8, 1.0, 1.5 and 2.0 mL, diluted with methanol to scale, shaken well, and injected 10μL. Linear regression was performed with the peak area (Y) as the ordinate and concentration (X) as the absciss coordinate. The regression equation, correlation coefficient (R) and linear range were  $Y=1.159 \times 10^7 X - 1.665 \times 10^6$  ( $R^2=0.9993$ ), and the linear range was 2.16~43.26 μg·mL<sup>-1</sup>.

### 3.3. Precision Test

10 μL of reference solution was absorbed accurately, and the sample was repeated for 6 times according to the chromatographic conditions under "2.3". The peak area was recorded and the RSD was calculated. The results showed an RSD of 0.67% (n=6). The precision of the instrument is good.

### 3.4. Repeatability Test

Samples of ZYP (Lot Number. S00033) were taken and ground into fine powder. Six samples of the test solution were prepared in parallel according to item "2.5", and samples were injected under item "2.3" for detection. The results showed that the average content of 5-HFM was 0.21% and the RSD was 0.97% (n=6). The results showed that the method had good repeatability.

Table 1. Determination results of sample recovery.

composition	Sample weight /g	Content in sample /mg	Amount added /mg	Measured quantity /mg	recovery /%	Average recovery /%	RSD/%
5-HFM	0.1012	0.2156	0.2163	0.4314	99.9	99.4	0.58
	0.0996	0.2122	0.2163	0.4251	99.2		
	0.0999	0.2127	0.2163	0.4234	98.7		
	0.1008	0.2146	0.2163	0.4318	100.2		
	0.1001	0.2133	0.2163	0.4249	98.9		
	0.0995	0.2120	0.2163	0.4262	99.5		

### 3.5. Recovery Test

As shown in Table 1, six copies of ZYP (Lot Number. S00033) with known content were accurately weighed, 0.1 g for each, and 1 mL of reserve solution of reference substance with concentration of  $0.2212 \text{ mg} \cdot \text{mL}^{-1}$  was added to each, and the test product solution was prepared according to the method of "2.5", and the chromatographic conditions under "2.3" were determined. The calculated recoveries were 98.7%-100.2% with RSD < 2%.

### 3.6. Stability Test

One portion of the test solution prepared according to "2.5" was injected for detection at 0 h, 1 h, 3 h, 7 h and 9 h respectively. The peak area was recorded, and the calculated RSD was 0.74%. The results showed that the test solution was basically stable within 9 h.

### 3.7. Sample Determination

The samples of ZYP with Lot Number S00033, T00058 and T00061 were prepared in parallel according to the method of "2.5" and the chromatographic conditions of "2.3" were determined. The content of 5-HMF in three batches of ZYP was calculated. The results are shown in Table 2.

**Table 2.** Determination of 5-HMF in 3 batches of samples.

Lot Number	Content in sample / ( $\text{mg} \cdot \text{g}^{-1}$ )
S00033	2.13
T00058	3.07
T00061	2.80

## 4. Discuss

In recent years, the safety of drugs has attracted more and more attention. According to the statistics of the national adverse drug reaction monitoring network, the number of adverse drug reactions time showed an increasing trend from 1990 to 2020. Therefore, effective control of harmful components in Chinese patent medicine has become the focus and hot spot of current research. Studies have shown that 5-HMF has a variety of pharmacological activities, but its obvious toxicity at a certain dose cannot be ignored. Therefore, 5-HMF is defined as a substance with dual effects of pharmacological activity and toxicity.[9-10].

5-HFM is mainly an aldehyde compound produced by the dehydration of monosaccharide compounds such as glucose under high temperature or weak acid conditions. Its chemical properties are relatively active, with high reactivity and polymerization ability [11]. It can form a variety of derivatives through oxidation, hydrogenation and condensation reactions. Therefore, it has low cytotoxicity and low mutagenicity to human cells [12].

As a sugar degradation product, 5-HFM has many factors affecting its content due to its poor stability. In recent years, there are many reports on the determination of 5-HMF in Chinese patent medicine preparations and Chinese herbal

pieces. In this study, 5- HFM in ZYP was used as the research object, and a method for the determination of 5- HFM by HPLC was established. Through literature review and previous experiments, it was determined that the extraction efficiency of ZYP was the highest when 10% methanol was used for 30 min. The absorption of 5-HMF was the highest at 284 nm [13, 14]. Therefore, the extraction method and detection wavelength were determined. From the results of methodological investigation, 5-HMF showed a good linear relationship with the peak area in the concentration range of 0.0200-0.4002  $\mu\text{g}$ , the correlation coefficient  $R^2=0.9993$ , and the results of specificity, precision, repeatability, average recovery, durability and stability test were qualified. HPLC method is not only sensitive and accurate, but also easy to operate. It can be used for the determination of 5-HMF, and has a certain guiding role for the quality control of ZYP [15]. The components of traditional Chinese medicine compound preparation are complex, which can not be evaluated and analyzed by one or several index components. It is not comprehensive, because it is often a comprehensive process, so it needs further study.

At present, most studies on the pharmacological activity of 5-HMF are limited to the cellular level. Whether it is ultimately harmful to the human body depends on different exposure routes, exposure doses and frequencies. It is necessary to conduct in-depth toxicity and limit studies on 5-HMF and its related products, which has practical and guiding significance for the quality control and safety application of foods and drugs containing 5-HMF.

## 5. Conclusion

The components of traditional Chinese medicine compound preparation are complex, which can not be evaluated and analyzed by only one or several index components. It is not comprehensive, because it is often a comprehensive process. However, ZYP is suitable for special population and may have potential harm. The author thinks that it is of certain significance to establish a limit test for 5-HMF or as an index in the quality control of ZYP to ensure its quality and clinical medication safety.

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