# Analytical Methods

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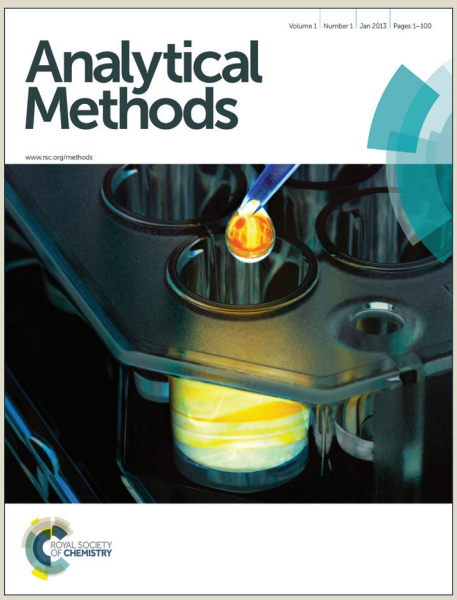





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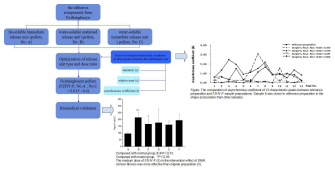

An integral evaluation method for the synchrony of drug release based on the mathematics set was developed for guiding the preparation of multi-component TCM.

# An integral evaluation method for the synchrony of drug release based on the mathematics set in guiding the preparation of a multi-component traditional Chinese medicine

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**Running title: An integral evaluation method for the synchrony of drug release**

***Abstract***

The quantitative characterization and evaluation of the synchrony of multi-component release behavior are bottlenecks urgently needed to solve in the studies on multi-component release kinetics and the quality control of multi-component traditional Chinese medicine (MCTCM). MCTCM is from the original prescription and preparation. At present, the evaluation of MCTCM is generally full use of pharmacodynamics evaluation. But it is lack of convenience and can not clearly reveal the correlation between the whole dynamic process of drug release and *in vivo* absorption. It is also very limited for the control of preparation. In addition, there are various types of effective ingredients in MCTCM, which exist distinct differences in physicochemical properties. These differences may lead to asynchrony of drug release between MCTCM and original preparation. So that they thereby directly affect the efficacy. However, in this issue through the pharmacodynamics evaluation we can't find the core reason. The papers reported were confined to studying the release characteristics of one or a few components in MCTCM and the drug release evaluation method reported was too macroscopic to specifically identify the components which caused the integral asynchrony.

In this paper, in order to reveal the MCTCM release synchronous characteristics, Fuzhenghuayu Capsules selected as the model, an original preparation, the integral release evaluation method based on the mathematics set was established in guiding the preparation of MCTCM. This method can not only be used in evaluating release characterization of MCTCM by the parameter of asynchronous coefficient, but also

be used in adjusting the dose of release unit by the relative error parameter. The results demonstrated that this evaluation method was feasible, stable and reproducible. And the Fuzhenghuayu Pellets guided by this method were showed release synchrony and similar pharmacodynamics with the original capsules, and the drug release mechanism was mainly frame erosion. Through this study, we could then evaluate the quantifiable release characteristics of MCTCM and it could apply an integral synchronous evaluation method for MCTCM.

***Keywords:*** drug release set; evaluation method of the synchrony; drug release; chromatographic fingerprint; Multi-Component Traditional Chinese Medicine

## *1. Introduction*

Currently, multi-component traditional Chinese medicine (MCTCM) is from an effective and classical formulation, which has clear active substances in the clinic and confirmed by pharmacodynamics.[1] According to the characteristics of the different effective components in the formulation, the MCTCM is usually comprised of two or more types of release units, such as pellets, granules, microcapsules etc. Then release units were recomposed into the capsules. Moreover, through optimizing different release unit composition, MCTCM can produce similar or superior effect to the original preparation. Presently, optimizing release unit composition of MCTCM depended on an pharmacodynamics assessment, such as pharmacological effect intensity, the onset time and the continuing role of time, et al. It is available for ensuring the rationality of the preparation as an terminal evaluation. However, it can not describe the whole release behavior of MCTCM. Furthermore, it can not find the reason of asynchronous release behaviour due to the method of preparation, the different physicochemical properties of ingredients, et al. These factors may also directly affect the curative effect of the drug. Therefore, attention should be paid to the quantification of synchrony of multi-component release research in MCTCM.

At present, there are mainly following three aspects of release evaluation of MCTCM.[2-4] The first one is the evaluation method based on the chemical composition which is with one or a few indicator components quantitative analysis.[5-11] This method is simple, fast and strong representative, but it is only suitable for single component preparation. The second one is the evaluation method based on

pharmacological effect and biological effect, a biological potency measurement method, which is used for the terminal evaluation. [12-14] The third one is the fuzzy evaluation method based on component theory, including Quantified fingerprint method[15-17] and Kalman filter method[18-22] which was used in the evaluation of the synchrony of Yinqiaojiedu tablets[19], Tongxuanlifei pills [16], Dachuanxiongfang pellets, Shaoyaogancaofang pellets and Yuchangning pellets [1]. Although Kalman filtering method was reported, because of its Ultraviolet spectrophotometry method with detecting all absorption in only one wavelength as “component score”, this method was too general to be able to specify which components caused integral asynchrony. As a result, it failed to identify which component accounted for the overall asynchrony and also failed to provide an effective reference for the design of MCTCM.

Fuzhenghuayu Capsules (FZHY-C) was developed by Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine Institute of liver disease[23-28], which included *Radix Salviae Miltiorrhizae*, *Cordyceps brasiliensis*, *Semenpersicae*, *Fiveleaf Gynostemma Herb*, *Schisandra chinensis* and *Pine Pollen*. Through the further study on the material foundation of the prescription, the effective components after extracted and purified and dosage ratio were obtained clear, which included Cordyceps polysaccharide (240 $mg \cdot kg^{-1}$), Salvianolic acid (130 $mg \cdot kg^{-1}$), Cordyceps fat soluble components (0.1 $mL \cdot kg^{-1}$), Gynostemma pentaphyllum polysaccharides (7.3 $mg \cdot kg^{-1}$), Gynostemma pentaphyllum saponins (20 $mg \cdot kg^{-1}$) and Amygdalin (0.1 mg $\cdot kg^{-1}$). The more information about FZHY-C and liver fibrosis were

shown in *Supplementary Data 1*.

The change of material composition would lead to change the coexistence environment of different polar substances. It may lead to the asynchrony release between the original preparation and recomposed release unit. Therefore, three FZHY Pellets (FZHY-P) with different release behaviors were prepared using modern technology according to the physical and chemical properties of the effective components. And the integral evaluation method for the synchrony of drug release based on the mathematics set was established. Moreover, relative error ($\varepsilon$), asynchronous coefficient ($k$) and other parameters were established for achieving the synchrony of the drug release behavior compared with the original preparation and recomposed preparation, screening the drug release unit type and dose. Through the above research, it provided an effective evaluation tool of drug release for the quantitative characterization and the synchrony of MCTCM.

## *2. Materials and methods*

### *2.1 Chemicals and materials*

FZHY-C was purchased from Shanghai Huanghai pharmaceutical Co., LTD (Shanghai, China). FZHY-P were self-made, with fat-soluble immediate release unit (pellets) of 2.5% drug loading for fat soluble active components (including Cordyceps fat soluble components, Gynostemma pentaphyllum saponins and Amygdalin) and water-soluble immediate release unit (pellets) or water-soluble sustained release unit (pellets) of 33% drug loading for water soluble active components (including Cordyceps polysaccharide, Salvianolic acid and Gynostemma pentaphyllum

polysaccharides).The preparation of fat-soluble immediate release unit (type: pellets, No: **A**), water-soluble sustained release unit (type: pellets, No: **B**) and water-soluble immediate release unit (type: pellets, No: **C**) were showed in S*upplementary Data2*. All these pellets with different unit type and dose ratio were put into empty capsule shells.

Reference standards of Protocatechuic aldehyde (>98%) and salvianolic acid B (>98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). 50% total salvianolic acid extract (TSA) and 80% total gypenoside extract (TG) were obtained from Shanghai Huanghai Pharmaceutical Co., Ltd. (Shanghai, China). Dimethylnitrosamine (DMN) were provided by Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). 4-Dimethylamino benzaldehyde, Chloramines-T, Anhydrous ethanol, Xylene, formaldehyde, Pentobarbital Sodium, Isopropanol (Analytic grade), Concentrated hydrochloric acid were all obtained commercially by China Sinopharm Co., Ltd of Shanghai chemical reagent Company (Shanghai,China). Sodium Chloride Injection (0.9%) was bought by Anhui Shuanghe Pharmaceutical Co., Ltd (Anhui, China). Hydroxyproline (Hyp) was bought by Sigma-Aldrich Chemie GmbH (American). Perchloric acid was bought by Shanghai taopu Chemical plant (Shanghai, China). Phosphoric acid was excellent pure-grade and all other chemicals were analytical grade.Acetonitrile and methanol, both HPLC grade, were purchased from Merck (Germany). Pure water system (for HPLC/UP) was bought by Labconco Company (American).

***2.2 Dissolution test and preparation of reference solution and sample solution***

A DT800-Ls intelligent dissolution instrument was purchased from Tianjin university wireless power plants. The dissolution test of FZHY-C and FZHY-P was prepared in accordance with the China Pharmacopeia.[29]

For each batch of reference solution, 20 FZHY-C were accurately weighted, equivalent to 2.00 times of single capsule weight ( ± 0.1 mg), was “fully” dissolved in 250 mL of distilled water by dissolving for 4 hours. The rotational speed was set at 75 revolutions per minute , and the temperature was set at 37 ± 0.5°C. The solution (5 mL) was filtered through a PTFE syringe filter (Millipore, pore size 0.22 μm, diameter 13 mm) as the calibration sample with a gross concentration of 200%, which represented 2.00 capsule was “fully” dissolved in 250mL of dissolution medium，then dried on the water bath at 70 °C, dissolved in the mobile phase (1 mL), filtered through a 0.45 μm membrane, and analyzed by high performance liquid chromatography (HPLC). The 200% calibration sample was serially diluted with distilled water to generate the other calibration samples, whose gross concentrations were 25%, 50%, 75%, 100%, 125% and 150%.

For each batch of sample solution, 20 FZHY-P were accurately weighted, equivalent to 2.00 times of FZHY-P weight ( ± 0.1 mg) . FZHY-P sample solutions were collected at 10, 30, 60, 120 and 240 min throughout the experiment, and all were prepared and analyzed as described above. Following sample removal, the appropriate media (5 mL) was immediately replenished.

### *2.3 Instruments and chromatographic conditions*

An Agilent Technologies 1200 HPLC system equipped with Agilent G1329A

automatic temperature control autosampler and Agilent G1314B variable wavelength detector were used. Samples were separated using a Kromasil C18 column (250 mm × 4.6 mm, 5 μm). The mobile phase was composed of methanol (A), acetonitrile (B) and water containing 0.2% $H_3PO_4$ (C) and was delivered at a flow rate of 0.8 mL ·$min^{-1}$. A gradient program was used as follows: the initial elution condition at 0 min was A:B:C ( 0.2% : 0.3% : 99.5% , v/v) , then linearly changed to A:B:C ( 1.2% : 1.8% : 97.0% , v/v) at 30 min, A:B:C ( 2.0% : 3.0% : 95% , v/v) at 31 min, A:B:C ( 4.0% : 6.0% : 90% , v/v) at 55 min, A:B:C (12.0% : 18.0% : 70.0% , v/v) at 90 min, A:B:C ( 20% : 30% : 50% , v/v) at 140 min, A:B:C ( 0.2% : 0.3% : 99.5% , v/v) at 150 min. The detection wavelength was 280 nm and the column temperature was set at 30 °C.

Validation was performed by establishing precision, stability and repeatability for all characteristic peaks. Chromatographic profiles were obtained for mixed standards of protocatechuic aldehyde, salvianolic acid B, TSA, TG and the reference solutions using the chromatographic conditions described above.

Intra-day precision tests were performed by analyzing FZHY-C reference solutions during a single day and inter-day precision tests during 3 days. For precision tests, reference solutions were analyzed six times continuously. For repeatability tests, six independent reference solutions were prepared as described above. For stability tests, six independent reference solutions were prepared and analyzed at 0 h, 3 h, 6 h, 9 h, 12 h and 24 h following storage at −4° C.

***2.4 Establishment of the evaluation method for the synchrony of drug release based on the mathematic sets***

A drug release set is defined as the assembly of chemical compounds dissolved in a medium from the TCM preparation.[18] It can also be considered as the significant part of the basis for pharmacodynamics.

The standard chromatographic fingerprint is defined as the characteristic fingerprint of a drug release set for full drug release of an original preparation at the specified dose. When the reference and sample preparations display similar release behavior, it is defined as synchrony. Conversely, dissimilar release behavior is defined as asynchrony.

When FZHY-C was fully released , the drug release set was comprised of several components (corresponding subscript symbols as “n”), so the peak area of drug release set could be described as {$A_{s,1}$, $A_{s,2}$, ……, $A_{s,(n-1)}$, $A_{S,n}$ }. Supposing the number of FZHY-P which consists of different drug release units was defined as “p” and the sampling time point of its *in vitro* release was defined as “t”, then the peak area of each component obtained from the “p”$^{th}$ sample can be expressed as $A_{p,n,t}$.

2.4.1 Establishment of standard curve of reference preparation

The reference preparation was “fully” dissolved as the calibration sample and it was diluted to a series of concentrations, then the standard curve was constructed by plotting peak area ($A_{s,n}$) against time on the x and y axes respectively. The concentration of each component of sample preparation was obtained as followed. Firstly, the $A_{p,n,t}$ of sample preparation were inputted into the standard curve regression equation, then the relative release amount ($C_t$) of the drug release set was be obtained according to the dilution ratio.

2.4.2 Accumulative release of the drug release set

After obtaining the relative release amount ($C_t$) of each characteristic peak in the drug release set, the cumulative release degree ($Q_t$) could be calculated in accordance with the formula below, and the curve for drug release could be fitted. Finally, the release mechanism could be speculated.

$$Q_t=\{[C_t\times V_0+\sum(C_{t-1}\times V_i)]/M\}\times 100\%$$

In the equation, "*t* " represented sampling time point(*h*), "$Q_t$" represented cumulative release degree (*%*) at sampling time point, "$C_t$" represented the concentration of each characteristic peak (i.e. release quantity), "$V_0$" represented total volume of dissolution medium, "*Vi* " represented sampling volume, and "*M* " represented release quantity of reference preparation (i.e., the product of the reference "$C_t$" and "$V_0$").

2.4.3 The synchronous characterization of the drug release set

The information provided from the reference and the sample fingerprints at different time points were compared by using the software "Similarity evaluation system for chromatographic fingerprint of TCM" (2004, A Chinese Pharmacopoeia Commission). The parameter(*s*) was closer to 1, the reference and sample preparations displayed the better similar release behavior, the synchrony was the better. When comparing different FZHY-P samples, the better synchrony was defined as the earlier time points whose similarity was the more approaching to 1.

When the release of sample and reference preparations was synchronous, the difference of cumulative release degree for both should be tend to zero, i.e.,

$Lim\sum(Q_{p,n,i} - Q_{s,n})=0$. Hence, the absolute value of the difference was defined as "Error" and was shown as "$Q_\varepsilon$". The error of each component obtained from the "p"[th] sample could be expressed as $Q_{\varepsilon\,(p,n,t)\ t}$. $Q_{\varepsilon\,(p,n,t)}$ reflected the error between reference preparation and samples. In order to reduce the calculation error, and thus the synchrony of each compound in the drug release set could be evaluated by "relative error" ($\varepsilon_{p,n,t}$), i.e. $\varepsilon_{p,n,t} = Q_{\varepsilon\,(p,n,t)} \ / Q_{s,n}$.

The error curve was obtained by plotting sample points "t" on the x-axis against the relative error "ε" on the y-axis. The parameter $k_{p,n}$ (the slope of the error curve) reflected the change of curve and was defined as the asynchronous coefficient "*k*". The "*k*" for the reference and sample preparations was tested by a rank sum test and the difference of the overall location and shape distribution was estimated respectively. When no difference was observed, we could conclude that the complete release process was similar or equal between the sample and reference preparations.

### *2.5 Animals, Drug Administration, Sampling*

Eighty-five white Sprague-Dawley (SD) rats were obtained commercially from Chinese Academy of Shanghai Experimental Animal Center [Shanghai, China, certification NO: SCXK (Shanghai): 2007-0005]. They were kept in an environmentally controlled breeding room in Experimental Animal Center of Shanghai Traditional Chinese Medicine University (Shanghai, China) for one week before starting the experiments and fed with standard laboratory food and water ad libitum. The animal facilities and protocols were approved by the Institutional Animal Care and Use

Committee, Shanghai University of TCM. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animal (The National Academies Press, revised edition 2010).

Rats were divided into six groups as followed : normal group (n=10), model group (n=19), the original preparation FZHY-C group (n=14), the high dose of FZHY-P group (n=14), the medium dose of FZHY-P group (n=14) and the low dose of FZHY-P group (n=14). The rats of model group were intraperitoneal injected with 2 $mL \cdot kg^{-1}$ (weight) DMN (0.5%). The period of injection was lasted in 4 weeks. The first injection was taken in 2/3 of the full dose. The injection was taken once a day. It had the rest of 4 days after the continuous injection of 3 days. This period of injection was total 3 weeks. In the $4^{th}$ week, the injection was taken 1/2 the amount of the full dose in $1^{st}$ day and $3^{th}$ day of $4^{th}$ week and was stopped 1 time during the period. Then after 3 days, the injection was also taken the full dose one time. The normal group rats were also intraperitoneal injected with the same amount of sodium chloride injection as model group rats at the same time of model group.

Each group were orally given with the corresponding dose of an aqueous solution with 10 $mL \cdot kg^{-1}$ (weight). The dose of FZHY-C group was 6.318 $g \cdot kg^{-1}$ (weight). The dose of the FZHY-P was shown in Table 1S. Each group was given once a day and lasted 4 weeks. The normal group and the model group were given the same dose of drinking water.

After the end of the experiment, the rats were fasted, but not limited water, and they were weighed after 24 hours. Then rats were sacrificed, and the livers and

spleens were removed to weigh. And then about two 1.0cm × 0.8cm × 0.3cm size liver tissue was taken and fixed in neutral buffered formalin (10%).

### *2.6 Biomedical study*

Hydroxyproline(Hyp) content of the liver tissue was determined by Jamall methods.[30] Microfuge lite was purchased from Beckman Company. Vortex mixer (XW-80A) was obtained commercially by the Haimen Lindberg Instrument Manufacturing Co., Ltd. M5 Multifunctional microplate reader was bought by American Molecular Devices Company.

Approximately 100 mg of liver tissue was weighed, dried water out with a filter paper, then it was placed in a homogenate tube, added 1.5mL amount of saline in homogenizer, homogenized and transferred to ampoules. Homogenate tube was flushed by 1 mL saline. The ampoule was added 2.5 mL HCl before sealed . Then the sealed ampoule was put into the oven at 105 $^{o}$C to hydrolyze for 20 hours. Then the solution of hydrolyzate was filtered, 100 μL amount of which was plused into a new homogenate tube. Each specimen was duplicated twice and each one was dried in the oven at 40 °C.

0.2 mL amount of the chloramine-T working fluid and 1.2mL of the 50% isopropanol were added to the homogenate tube. Then it was stayed at 25 °C room temperature for 10 min. 1 mL of the ER working fluid was next added to it, i.e. the Euclidean liquid [Ehrlich's reagent solution, 25% (w / v) dimethylamino benzaldehyde and 27.3% (v / v) perchloric acid solution in isopropanol]. After mixed with shaking, it was set at 50 °C warm bath for 90 min. OD value was measured at the wavelength of 558 nm after distilled water was used to zero. The OD value of each tube and its

duplicated tube were taken the mean, Hyp content was calculated based on the standard curve, and corrected by precise liver tissue wet weight .

Standard curve was drawn as followed Table 2S. OD value was measured at the wavelength of 558 nm after distilled water was used to zero. Standard curve was drawn according to the standard concentration.

### *2.7 Statistical analysis*

Standard chromatographic fingerprint was generated by using the software "Similarity evaluation system for chromatographic fingerprint of TCM" (2004, A Chinese Pharmacopoeia Commission). Differences between parameters for each group of drug release were tested by rank sum test by using SPSS 18.0 software for Windows (MapInfo Corporation, Troy, NY, USA).

The data was expressed as means $\pm$ *SD* in pharmacodynamic evaluation. One-way analysis of variance (ANOVA) and further *LSD-t* multiple comparisons were also employed by SPSS 18.0. A *P*-value below 0.05 or 0.01 was taken to indicate significant difference between data means.

## *3. Results and discussion*

### *3.1 Validation of chromatography method*

The results showed that linear relationship of each characteristic peak in the standard fingerprint had a good linear relationship. Regression equations and correlation coefficients of characteristic peaks are shown in Table 1. Table 1 also listed the results of precision, repeatability and stability tests. For intra-day precision analysis, the data showed that the relative standard deviations (*RSDs*) of retention

time and the peak area were less than 0.01% and 3.67% respectively. Meanwhile those for inter-day variation were less than 0.04% and 3.80%, respectively. The results for repeatability were less than 0.03% with respect to retention time and 5.93% for peak area. For the stability tests, the *RSDs* for retention time and peak area were less than 0.03% and 4.15% respectively, indicating that the sample was stable over 24 h. The validation data indicated that the analytical method was specific, sensitive and stable.

### *3.2 Standard chromatographic fingerprint of the drug release set*

3.2.1 Acquisition of standard chromatographic fingerprint

Characteristic fingerprint chromatograms of 10 batches FZHY-C were shown in Fig.1. This data was generated from the reference standard fingerprint chromatogram by using the software, a similarity evaluation system for chromatographic fingerprint of TCM.

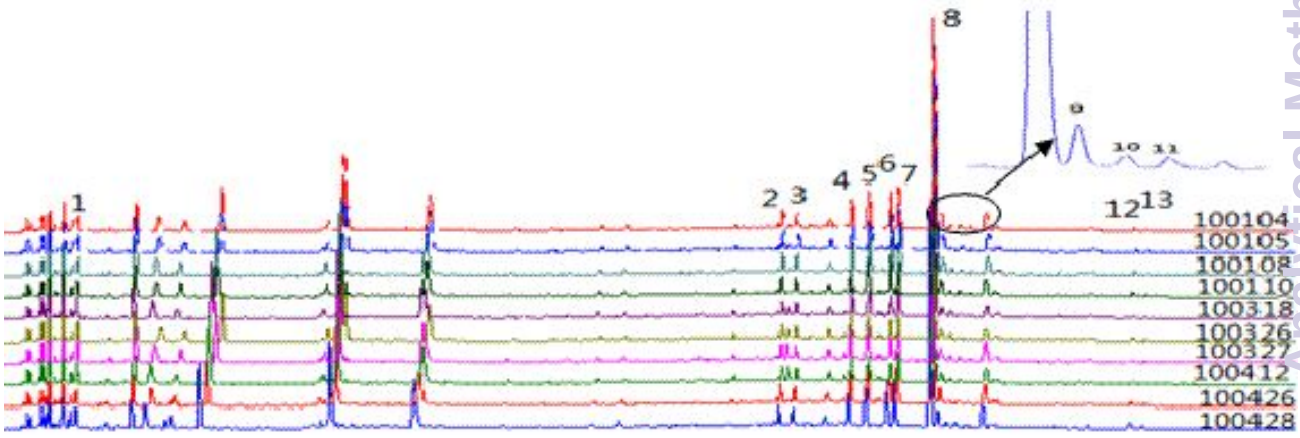


Fig. 1 HPLC fingerprint chromatograms of 10 batches FZHY- C.

3.2.2 Characteristic peak identification and similarity evaluation

13 Characteristic peaks were identified, in which the fourth peak (94.33 min) was

the reference peak (4th s). Others had the following relative retention times: 0.07 min (1st), 0.96 min (2nd), 0.98 min (3rd), 1.00 min (4th s), 1.02 min (5th), 1.04 min (6th), 1.06 min (7th), 1.09 min (8th), 1.10 min (9th), 1.12 min (10th), 1.14 min (11th), 1.16 min (12th) and 1.23 min (13th). From the Fig.2, based on comparisons of the mixed standards of protocatechuic aldehyde and salvianolic acid B, total salvianolic acid extract (TSA), total gypenoside extract (TG) and FZHY-C reference preparation, peak numbers 1, 5, 6, 7, 8, 9, 10, 11 and 13 were from TSA, peak numbers 2, 3, 4, 12 were from total gypenoside, and peak 8 was salvianolic acid B. The average similarities of the 10 randomly-selected samples were >0.96.

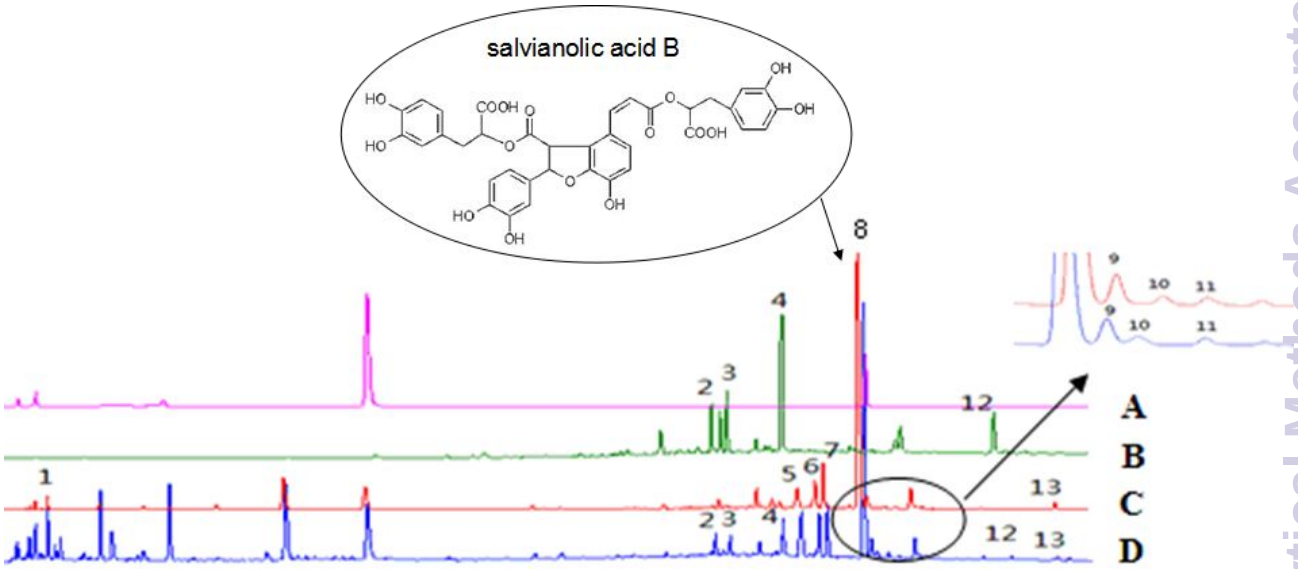


Fig. 2 The characteristic peaks in FZHY-C fingerprint chromatogram. (A: mixed standard of protocatechuic aldehyde and salvianolic acid B. B: total salvianolic acid extract, TSA. C: total gypenoside extract, TG. D: FZHY-C fingerprint chromatogram).

3.2.3 Determination of standard chromatographic fingerprint

The reference fingerprint "R" generated by the similarity evaluation system for chromatographic fingerprint of TCM software was regarded as the standard fingerprint chromatography of the evaluation method. The drug release set for the standard chromatogram was formed by characteristic peak areas and could be expressed as: {$A_{s,1}$, $A_{s,2}$, $A_{s,3}$, $A_{s,4}$, $A_{s,5}$, $A_{s,6}$, $A_{s,7}$, $A_{s,8}$, $A_{s,9}$, $A_{s,10}$, $A_{s,11}$, $A_{s,12}$, $A_{s,13}$} = {79.2, 181.6, 20.7, 87.8, 168.8, 351.1, 433.8, 3949.9, 154.6, 96.4, 50.1, 31.1, 81.3}.

### *3.3 Optimization of release unit type of FZHY-P by using the synchronous evaluation method based on the mathematics set*

For optimizing the release unit type of FZHY-P, the release unit composition was designed. Sample 1 was contained of fat-soluble immediate release unit (A, 0.015 g) and water-soluble sustained release unit (B, 0.285 g). Sample 2 was contained of A (0.015 g) and water-soluble immediate release unit (C, 0.285 g). The peak area of the drug release set in both standard and sample fingerprint chromatograms were recorded in the release experiment described in sections 2.2. The chromatographic conditions described in section 2.3. Each experiment was repeated three times. The parameter (*s*) of the reference preparation and samples are shown in Table 2, relative errors are shown in Fig. 3 and the statistical results are shown in Table 3.

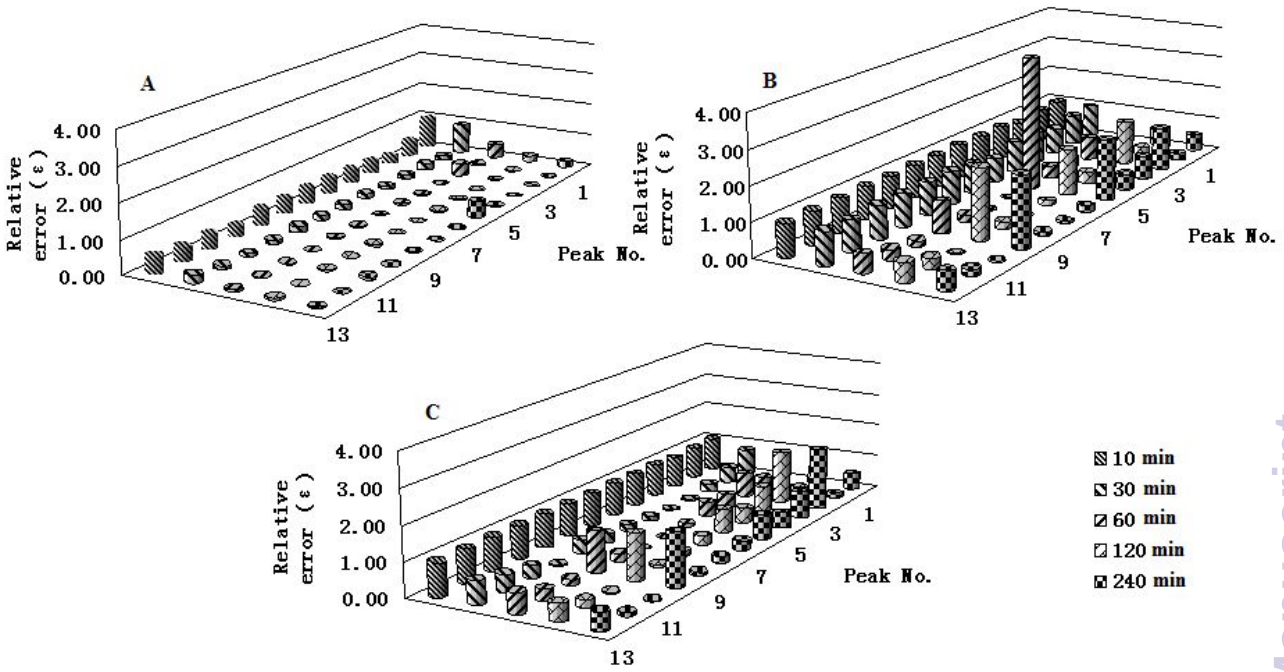


Fig. 3 The relative error (ε) of reference preparation and FZHY-P sample preparations. (A. reference preparation; B. sample 1; C. sample 2).

The average similarity (*s*) of both samples 1 and 2 were less than 0.9, as shown in Table 2, indicating a big difference between the two samples and the reference preparation, especially within 60 minutes. One explanation could be the sustained release effect which was caused by the sustained release pellets in sample 1. After 60 minutes, the similarity had become much closer to 1, which indicated that the release degree of samples was becoming more synchronous to the reference preparation. We further observed that the peak numbers 3, 6 and 10 in sample 1 and 2 showed the biggest change in relative error ($\varepsilon$) by comparison with the reference preparation (Fig. 3). It explained that the asynchrony was from the above three peaks and next we should pay attention to adjusting the release unit ratio to improve synchrony. From Table 3, there were significant differences ($P < 0.05$) between

sample 1 and the reference preparation, but not sample 2.So the sample 2 was more similar than sample 1 to the reference preparation.

In summary, this data showed that it was suitable that FZHY-P was composed of A and C.

### *3.4 Optimization of dose ratio of FZHY-P by using the synchronous evaluation method based on the mathematics set*

While some components such as peak number 6 and 10 were found to affect the synchrony of the overall sample. Since these components were mostly derived from TSA, TSA was one of water soluble active component, so we adjusted the water-soluble immediate pellets dose to ensure that the pellets was similar to the reference preparation in release character.

For optimizing the ratio dose of FZHY-P, all the A were 0.015 g. The C of Sample 3, Sample 4, Sample 5, Sample 6 were 0.200 g, 0.250 g, 0.285 g, 0.400 g, respectively. The similarity of the reference preparation and samples are shown in Table 2, relative errors are shown in Fig. 4 and the statistical results are shown in Table 3.

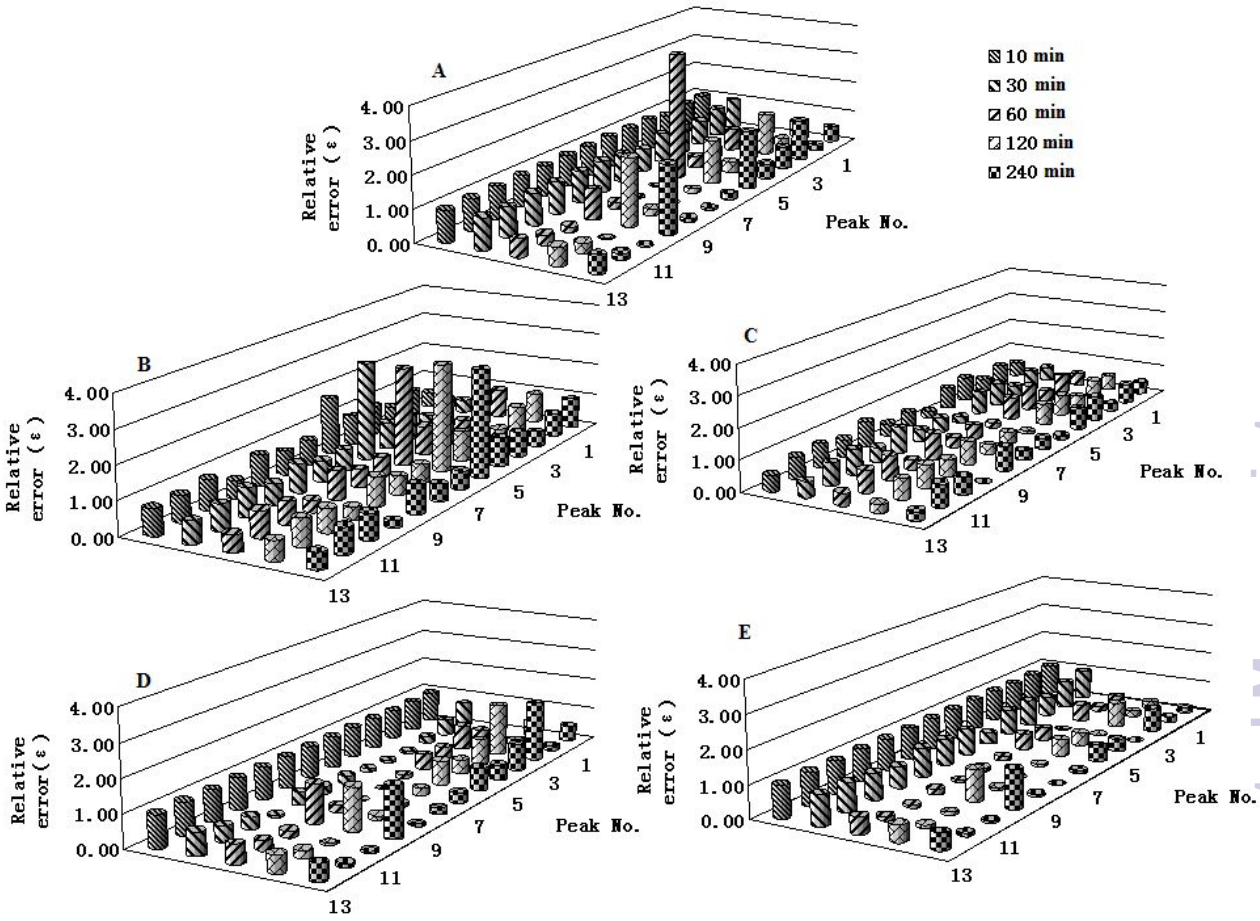


Fig. 4 The relative error ($\varepsilon$) of reference preparation and FZHY-P sample preparations. (A. reference preparation; B. sample 3; C. sample 4; D. sample 5; E. sample 6).

As the average similarity of the samples was less than 0.9 (Table 2), indicating that the synchrony of all the samples had big differences when compared to the reference preparation. In Fig. 4, with the increase of C dose from sample 3 to sample 6, the parameter ($\varepsilon$) gradually improved, which indicated that the release unit ratio was predictable expediently by this evaluation method based on the mathematical set. Meanwhile, when the dose of C increased, the difference of parameter ($k$) became smaller compared with the reference preparation from sample 3 to sample 6

(Table 3). Through the analysis of variance, sample 3 had significant difference ($P < 0.05$), and sample 6 was the most similar ($P = 0.42$). With the exception of sample 6, the parameter ($k$) of the other samples all had a significant difference ($P < 0.05$, $P < 0.01$). So sample 6 was more similar than other samples to the reference preparation.

In summary, this data showed that the dose ratio of the C and A were 0.4 : 0.015, suggesting that the release character of FZHY-P was similar to the reference preparation.

At the same time, we observed that statistical method could affect the accuracy of the evaluation method. Initially we used the t-test[22] to assess the parameter($k$) between each sample and reference preparation. The results showed that samples 1, 2, and 3 had no distinct difference ($P > 0.01$). In contrast, by the rank sum test[31-34], samples 3, 4 and 5 were significantly different when compared to the reference preparation ($P < 0.01$). We observed that the variation in the statistical methods was due to the data normality and homogeneity of the variance test. It was assessed that the data of the reference preparation and samples did not meet the requirements of the t-test for data normality, therefore two kinds of statistical methods produced different results. Unlike the t-test, the rank sum test was more suited to non-normally distributed data and was not restricted by the overall distribution. Due to the consideration of the overall position and peak shape, the Kolmogorov-Smirnov z test was more creditable than Wilcoxon test used commonly[35-39]. Fig. 5 could demonstrated intuitively that the asynchronous coefficients of sample 6 was

closer to reference preparation in the shape and position than other samples.

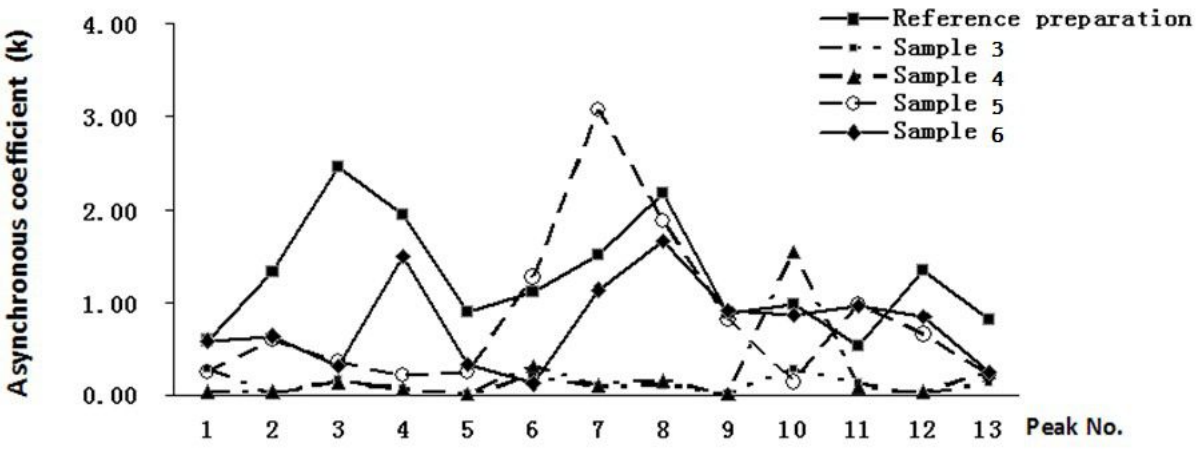


Fig. 5 The comparation of asynchronous coefficient of 13 characteristic peaks between reference preparation and FZHY-P sample preparations.

### *3.5 Drug release mechanism of FZHY-P*

There were mainly many release mechanism models for drug release evaluation[5,29], such as Zero-order drug release equation ($Q=Kt$), One release equation [$Q=1-exp(-Kt)$], Hixcon-Crowell Dissolution equation ($1-Q^{1/3}=1-Kt$), Higuchi planar diffusion model equation ($Q=Kt^{1/2}$) and Baker-Lonsdale spherical diffusion model equation {$3/2[1-(1-Q)^{2/3}]-Q=Kt$}.

Model fitting was done according to the accumulative release rate of each component, then it was verified by Ritger-Peppas equation, and finally the drug release mechanism of each component in FZHY-P was got. The curve fitting results of sample 6 are shown in Table 4. The results showed that the model fitting of the Hixcon-Crowell dissolution equation was the best for the vast majority of components in FZHY-P except the third peak, which indicated that the drug release mechanism of FZHY-P was mainly in corrosion process.

According to Ritger-Peppas[40], for ball type preparation, the meaning of exponent of time 't' is as followed. Drug release is mainly in the Fickian diffusion when n ≤ 0.43. When 0.43 < n < 0.85, drug release is for non-Fickian diffusion (drug diffusion with skeleton solutional phase coordination). When n ≥ 0.85, drug release mode belongs to mainly frame erosion. In this paper, the evaluation method based on the mathematics set was used to represent the synchronicity of drug release on FZHY-P. Through the validation of this model equations, it was shown that the third peak's n value was greater than 0.43 and less than 0.85, which release mechanism belonged to the diffusion dissolution synergy, meanwhile the remaining 12 characteristic peaks of the release mechanism were mainly frame erosion.

### *3.6 Evaluation of efficacy against liver fibrosis*

The fresh spleen of each group was observed (including the color, texture, surface smoothness). The liver of normal group was red, tender, soft and had smooth surface and the edge was sharp, and the spleen was dark red and medium texture. However, the liver volume of model group was significantly reduced and the liver was dark red, hard texture, rough surface and the edge of it was blunt. The spleen volume of model group was also largely increased. Compared with the model group, the livers of other drug groups (except the low dose of FZHY-P group) were better in color and texture than model's, and spleens were also narrowed in some degree.

Compared with the normal group, the body weight, liver weight and liver weight / weight of the model group were significantly lower ($P < 0.01$), spleen weight and spleen weight /weight of model group increased significantly ($P < 0.01$). Compared with the model group, the weight of original preparation FZHY-C group,

the high dose, medium dose of FZHY-P groups were higher than the model group and the medium dose of FZHY-P group had significant higher($P < 0.05$). Compared with the model group, the body weight and liver weight of the low dose of FZHY-P group decreased, and its of medium dose of FZHY-P had statistically decreased ($P < 0.01$). The results were showed in Table 3S.

The Hydroxyproline content of each groups were also tested. Compared with the same period of normal group, Hyp content of model group liver tissue was significantly higher ($P < 0.01$). Compared with model group, Hyp content of the original preparation FZHY-C group and medium dose of FZHY-P group liver tissues had decreased significantly ($P < 0.05$). The results were showed in Table 3S and Fig .6.

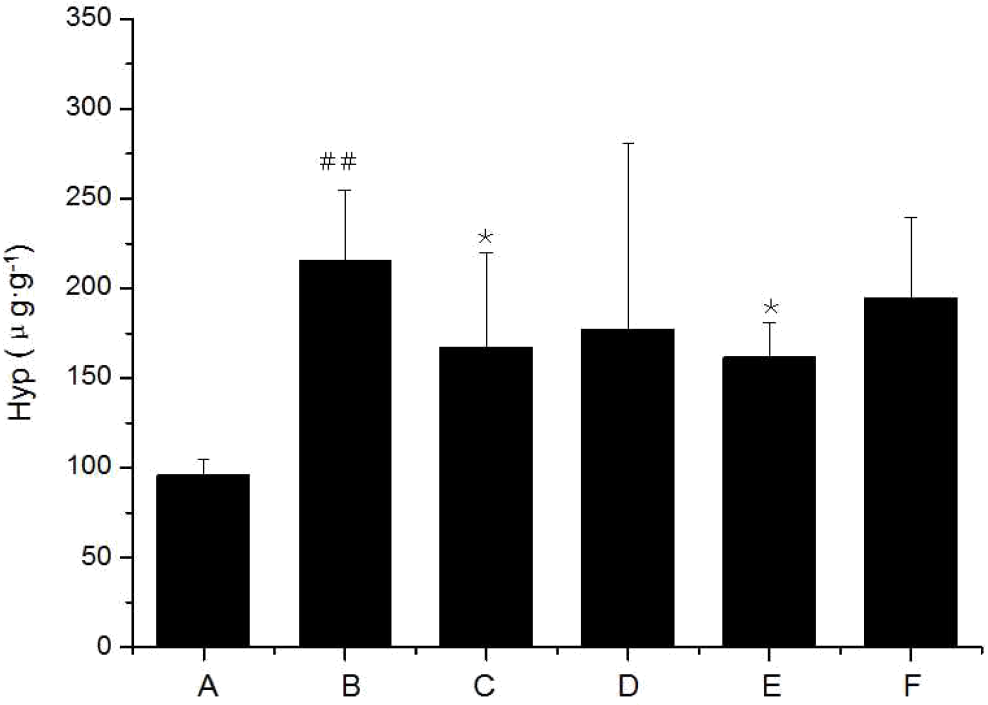


Fig. 6 Hyp content of each group rats' liver tissue. (A. Normal group, N=10; B. Model group, N=19; C. FZHY-C group, N=14; D. FZHY-P high dose group, N=14; E.

FZHY-P medium dose group, N=14; F. FZHY-P lose dose group, N=14)

Compared with normal group, $^{\#\#}P<0.01$; Compared with model group, $^{*}P<0.05$.

The above results had shown that the medium dose of FZHY-P in the intervention effect of DMN rat liver fibrosis was more effective than original preparation. In this study, the content of Hyp in liver tissue, as the main pharmacodynamic indicator could evaluated the reasonableness of the FZHY-P. The results demonstrated the same effect as the original preparation, indicating that the evaluation method for the synchrony of drug release based on the mathematics set was reasonable and convenient in guiding its preparation .

## *4. Conclusions*

Using the original preparation FZHY-C as the model, an integral evaluation method for the synchrony of drug release based on the mathematics set for MCTCM has been established. This method can not only be used in evaluating release characterization of MCTCM by the parameter of asynchronous coefficient, but also be used in adjusting the dose of release unit by the relative error parameter.

The results demonstrated that this evaluation method was feasible, stable and reproducible. And the FZHY-P guided by this method showed release synchrony and similar pharmacodynamics with the original preparation, and the drug release mechanism was mainly frame erosion. Through this study, we could then evaluate the release characteristic of MCTCM and it could apply an integral synchronous evaluation method for multi-component dissolution / release.

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**Table legends**

Table 1 Regression equations, related coefficients of each characteristic peak, inter-day and intra-day precision, repeatability and stability.

| Peak No. | Retention time | Regression equation | Retention time (% *RSD*) | | | | | Peak Area(% *RSD*) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Related coefficient (*r*) | Intra-day precision | Inter-day precision | Repeata-bility | Stability | Intra-day precision | Inter-day precision | Repeata-bility | Stability |
| 1 | 6.445 | Y =0.0855 X -0.6864 | 0.978 0 | 0.00 | 0.01 | 0.00 | 0.33 | 1.89 | 3.58 | 5.93 | 4.15 |
| 2 | 91.002 | Y =0.0332 X +0.0175 | 0.999 3 | 0.00 | 0.00 | 0.00 | 0.00 | 1.55 | 1.64 | 0.00 | 1.82 |
| 3 | 92.809 | Y =0.2501 X +0.2804 | 0.984 7 | 0.00 | 0.04 | 0.00 | 0.02 | 2.28 | 2.66 | 1.60 | 2.05 |
| 4 | 94.811 | Y =0.0640 X -0.1081 | 0.999 6 | 0.00 | 0.04 | 0.00 | 0.02 | 2.36 | 2.50 | 4.39 | 0.71 |
| 5 | 96.798 | Y =0.0264 X +0.0184 | 1.000 0 | 0.00 | 0.02 | 0.00 | 0.04 | 0.44 | 0.61 | 2.72 | 1.41 |
| 6 | 98.659 | Y =0.0962 X -0.3394 | 0.999 2 | 0.00 | 0.00 | 0.00 | 0.03 | 3.11 | 3.29 | 2.49 | 0.54 |
| 7 | 100.231 | Y =0.0129 X +0.0627 | 1.000 0 | 0.00 | 0.00 | 0.00 | 0.03 | 0.62 | 0.70 | 2.99 | 0.16 |
| 8 | 102.974 | Y =0.0015 X +0.0069 | 1.000 0 | 0.01 | 0.03 | 0.00 | 0.08 | 0.26 | 0.31 | 5.20 | 0.16 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9 | 104.761 | Y =0.0360 X +0.0038 | 1.000 0 | 0.00 | 0.00 | 0.00 | 0.03 | 0.36 | 0.34 | 2.33 | 2.22 |
| 10 | 106.601 | Y =0.0674 X -0.3836 | 0.997 4 | 0.01 | 0.04 | 0.03 | 0.02 | 0.74 | 0.89 | 3.48 | 2.52 |
| 11 | 108.017 | Y =0.1186 X +0.0020 | 1.000 0 | 0.00 | 0.02 | 0.00 | 0.04 | 1.24 | 1.58 | 2.13 | 1.82 |
| 12 | 110.016 | Y =0.2075 X -0.0666 | 0.999 7 | 0.00 | 0.02 | 0.00 | 0.04 | 3.15 | 3.68 | 0.96 | 0.76 |
| 13 | 116.826 | Y =0.0731 X +0.0845 | 0.999 8 | 0.00 | 0.03 | 0.00 | 0.05 | 3.67 | 3.80 | 1.66 | 3.90 |

1

2

Table 2 Similarity (s) of release of samples and reference preparation.

| Sample No. | s | | | | | |
|---|---|---|---|---|---|---|
| | 10min | 30min | 60min | 120min | 240min | AVERAGE |
| Reference | 0.872 | 0.926 | 0.951 | 0.974 | 0.976 | 0.940 |
| Sample 1 | 0.105 | 0.402 | 0.936 | 0.953 | 0.943 | 0.668 |
| Sample 2 | 0.000 | 0.906 | 0.937 | 0.979 | 0.978 | 0.760 |
| Sample 3 | 0.504 | 0.697 | 0.783 | 0.851 | 0.900 | 0.747 * |
| Sample 4 | 0.650 | 0.794 | 0.887 | 0.937 | 0.952 | 0.844 |
| Sample 5 | 0.000 | 0.906 | 0.937 | 0.979 | 0.978 | 0.760 |
| Sample 6 | 0.000 | 0.916 | 0.965 | 0.972 | 0.986 | 0.768 |

Compare to the reference preparation, *$p$< 0.05.

Table 3 The statistical results of asynchronous coefficient (*k*) of FZHY-P and reference preparation.

| No. | k | | | | | | |
|---|---|---|---|---|---|---|---|
| | Reference preparation | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 | Sample 6 |
| 1 | 0.579 | 0.248 | 0.232 | 0.269 | 0.032 | 0.232 | 0.581 |
| 2 | 1.330 | 0.564 | 0.594 | 0.021 | 0.033 | 0.594 | 0.627 |
| 3 | 2.462 | 0.173 | 0.365 | 0.148 | 0.139 | 0.365 | 0.303 |
| 4 | 1.934 | 0.134 | 0.1984 | 0.051 | 0.065 | 0.198 | 1.499 |
| 5 | 0.900 | 0.301 | 0.239 | 0.013 | 0.010 | 0.239 | 0.318 |
| 6 | 1.117 | 0.247 | 1.262 | 0.183 | 0.307 | 1.262 | 0.126 |
| 7 | 1.508 | 1.097 | 3.078 | 0.083 | 0.107 | 3.078 | 1.126 |
| 8 | 2.184 | 1.074 | 1.876 | 0.101 | 0.156 | 1.876 | 1.661 |
| 9 | 0.879 | 0.680 | 0.800 | 0.020 | 0.024 | 0.800 | 0.917 |
| 10 | 0.982 | 0.275 | 0.1382 | 0.269 | 1.539 | 0.138 | 0.864 |
| 11 | 0.534 | 1.397 | 0.972 | 0.119 | 0.081 | 0.972 | 0.965 |
| 12 | 1.333 | 0.519 | 0.660 | 0.012 | 0.032 | 0.660 | 0.833 |
| 13 | 0.800 | 0.211 | 0.210 | 0.140 | 0.232 | 0.210 | 0.239 |
| K-S Z | | 1.569* | 1.177 | 2.550** | 2.353** | 1.177* | 0.981 |

Compare to the reference preparation, *$P < 0.05$

Compare to the reference preparation, **$P < 0.01$

Table 4 The drug release curve fitting results of sample 6

| Peak NO. | Model | Fitting equation | Correlation coefficient(*r*) |
|---|---|---|---|
| 1 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.6395t$ | 0.977 1 |
| 2 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.4945t$ | 0.989 6 |
| 3 | Higuchi Diffusion equation | $Q=0.1039t^{1/2}$ | 0.955 3 |
| 4 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.2306t$ | 0.984 2 |
| 5 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-4.6473t$ | 0.941 1 |
| 6 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-1.4185t$ | 0.948 1 |
| 7 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.1969t$ | 0.967 6 |
| 8 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.8478t$ | 0.970 8 |
| 9 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.5575t$ | 0.980 3 |
| 10 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-1.5170t$ | 0.996 4 |
| 11 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.6989t$ | 0.990 2 |
| 12 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.8494t$ | 0.974 0 |
| 13 | Hixcon-Crowell Dissolution equation | $(1-Q)^{1/3}=1-0.4785t$ | 0.994 3 |

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