

Application of Design of Experiment and Design Space (DOE-DS) Methodology for the HPLC Separation of Panax Notoginseng Saponins

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Abstract: Panax Notoginseng Saponins (PNS), extracted from the roots of the common TCM Panax notoginseng (Burk.) F.H.Chen, consist of notoginsenoside R₁, ginsenoside Rg₁, Re, Rb₁, Rd and many other chemicals which haven't been identified. Due to its popular pharmacological effects, it is of importance to control the quality. This study combines design of experiments and design space methodology to optimize the HPLC separation of PNS. Three common chromatographic parameters (i.e. the temperature, the initial proportion of acetonitrile and the gradient slope) were selected to construct a Box-Behnken design which consisted of 17 experiments. A quadratic model that was built from the experimental result was used to construct the design space. The optimal separation was predicted at temperature 20°C, with a gradient starting at 15% of acetonitrile and a gradient slope of 0.55%/min. Accuracy profile approach was employed to validate the established HPLC method. The results clearly showed that quality by design methodology could be effectively applied to optimize the HPLC chromatographic conditions for the analysis of PNS.

Keywords: Panax notoginseng saponins, quality by design, design of experiments, design space, accuracy profile.

1. INTRODUCTION

Nowadays, though chemical drugs have a strong impact on the world's drug market, Traditional Chinese Medicine (TCM) still holds an important place to cure diseases in many Asian countries. The root of *Panax notoginseng*, called Sanqi or Tianqi in China, is a popular medical material which is well known for its efficacy in promoting blood circulation, removing blood stasis. And it is important to control the quality of PNS to achieve its clinical safety and efficacy for PNS is the main component in *Panax notoginseng*. Most of the studies about the quality control of PNS are based on HPLC, and the optimal chromatographic conditions were obtained using one factor at a time (OFAT) experiments. However, the OFAT method cannot reveal the interaction among different factors. Recently, Quality-by-design (QbD) concept advocated by ICH and FDA has been introduced into the fields of chromatographic analysis [1, 2]. QbD contained several parts, such as design of experiments (DoE) and design space (DS). Design of experiments (DoE) methodology was used to investigate the factors effects and the interactions between them by modifying multiple factors at a time [3]. Design space is defined as the "multidimensional combination and interaction of input variables that have been demonstrated to provide assurance of quality" [4]. Therefore, combined DoE with DS, a robust analytical method can be developed to satisfy the movement of HPLC parameters.

The main objective of this work is to develop a method for the separation of the 5 components in PNS by design of experiments and design space based on HPLC, and then the method was validated by accuracy profile methodology based on total errors theory [5].

2. MATERIALS

2.1. Reagents

Notoginsenoside R₁ reference standard was purchased from Nature Standard Biological Technology Co., Ltd. (lot:STA-25613013), ginsenoside Rg₁, Re, Rb₁ were purchased from Sichuan Weikeqi Biological Technology Co., Ltd. (lot: 110626, 110819, 110723, respectively), and ginsenoside Rd was purchased from Jiangxi bencaotiangong Science and Technology Co., Ltd.(lot:ROH-130912). The reagents were purchased as follows: both methanol HPLC grade and acetonitrile HPLC grade from Fisher scientific (Erembodegem,Belgium), distilled water.

2.2. Extraction

The sample Panax Notoginseng Saponins extraction was purchased from Jiangxi bencaotiangong Science and Technology Co., Ltd(lot:20130825).

2.3. Apparatus

Chromatographic separations were performed with a reversed phase C18 column (150 mm × 4.6 mm i.d.; particle size: 5µm), a HPLC agilent 1100 series separation module, coupled to a DAD spectrophotometric detector.

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3. EXPERIMENTAL

3.1. Sample preparation

The sample was dissolved to 10mL 70% methanol, filtered through a 0.45 μ m filter. Finally, 10 μ l of this solution were injected into the HPLC system.

3.2. Experimental design

DOE is considered a time-efficient and cost-efficient technique used to simultaneously identify the effects of multiple factors on results [6]. Box-Behnken Design was chosen to complete method optimization which was one of the design pattern of RSM (Response Surface Method). The method optimization involved 3 chromatographic factors: temperature, gradient slope and the initial concentration of acetonitrile and all of them was studied with 3 levels (see Table 1). 17 experiments of the three parameters have been tried according to the requirements of Box-Behnken Design.

3.3. Chromatographic conditions

The mobile phase consisted of acetonitrile and water. Experiments were carried out in gradient mode. The temperature of the column was adjust to 20°C, 24°C, 28°C, flow rate was 1 ml/min and the detection was performed at a wavelength of 203 nm. Peaks were fitted by Origin 8 software as some peaks may not be separated under some chromatographic conditions.

3.4. Method validation

Accuracy, precision, limit of detection, limit of quantitation, linearity and range is the common parameters to

estimate the method validation. The classical approaches are to investigate these parameters separately which need much time and resources. AOAC International defines a validated method as a method that is fit for its intended purpose and accuracy profile [7] is one of the methods. Accuracy profile has become an effective decision tool to assess the validity of analytical methods which based on the three most important validation parameters: trueness, precision and range. The validation of the method was done during three days to evaluate time-dependent intermediate precision. Four concentration levels were selected to establish the curve. Each level of the validation standards was analyzed independently in triplicates.

4. RESULTS AND DISCUSSION

4.1. Experiment design

The first step is to verify the validity of the model for each response. In total, 5 peaks were identified as Notoginsenoside R₁, ginsenoside Rg₁, Re, Rb₁, Rd (from left to right in the chromatogram). As depicted in Fig. (1), the adequacy between the sample retention times and the standard is good. Factors which have the highest effects on the response will be selected. Usually, the chromatographic resolution (Rs) between two adjacent peaks is modeled to assess separation, and the least Rs of two peaks was regarded as the critical peaks when there are many Rs in one chromatogram. The objective of optimization of the HPLC method is to let the critical Rs meet the Pharmacopoeia requirements. Besides, another objective is to shorten the running time (t_R). After comparing different models, the quadratic model was selected because it has the largest "Adjusted R-Squared" and the "Predicted R-Squared". The critical peaks are the ginsenoside

Table 1. Chromatographic parameters studied for the method optimization. (B%: the initial concentration of acetonitrile)

| Parameter | Levels | | |
|-----------------|--------|-----|-----|
| Temperature[°C] | 20 | 24 | 28 |
| Gradient slope | 0.4 | 0.6 | 0.8 |
| B[%] | 5 | 10 | 15 |

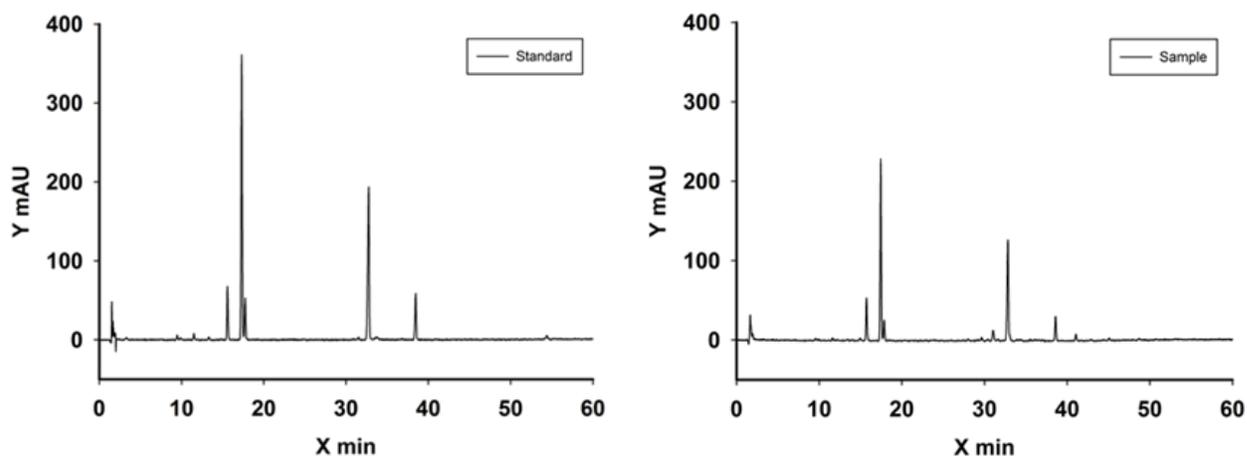


Fig. (1). The adequacy between the sample retention times and the standard ones is good.

R_{g_1} and ginsenoside R_e in the separation of *PNS*. These responses were modeled by quadratic equations (see Eq. 1)

$$R_s = 5.16 - 0.0629 \cdot T - 6.36 \cdot G + (6.40E-003) \cdot B + 0.0438 \cdot T \cdot G + (2.71E-007) \cdot T \cdot B + 0.025 \cdot G \cdot B - (3.12E-005)T^2 + 2.49G^2 - (1.22E-003) \cdot B^2 \quad (1)$$

where R_s represents the separation of the critical peaks. T (temperature), G (gradient), B are the factors of the design of experiments. Eq. 1 regard R_s as the response to build mathematic model, and t_R can also be seen as one response to build a quadratic model as R_s . The "Adjusted R-Squared" of the model is 0.987 and the "Predicted R-Squared" is 0.9975 indicating this model is of significance to navigate the design space. The DS corresponds to a region of the experimental domain where the probability to attain $R_s > 1.5$ and $t_R > b$ (b represents a certain time can be set by end user)

The overlay plot shows the Design Space of the method (see Fig. 2). The colored part represents the interaction of the two responses. The operating conditions changed included in the bright yellow zone will not cause the changing of the method. That is to say, the method is not needed to be validated under the condition. However, there will be some risk if the operating conditions changed included in the dark yellow zone. The dark yellow zone was built based on 95% confidence intervals. Besides, the optimal separation was predicted at 20°C, with a gradient starting at 15% of acetonitrile and a gradient slope of 0.55%/min from the Design Space(the blue dot in the Fig. 2). This optimal condition will be used at method validation.

4.2. Method validation

Trueness is related to the systematic error from its definition. It is expressed in relative bias(%) at each concentration level of the 3 repeats for the 3 days. Relative

bias was less than 5% for the 5 components (see Table 2, take notoginsenoside R_{g_1} as an example, the follows are the same) of the four levels showing the adequate trueness of the method except the least concentration level. Relative bias for the least concentration is more than 5% means the least concentration cannot satisfy the requirement of trueness.

Precision is related to the accidental error from its definition. It is expressed in relative standard deviation (RSD%) values for repeatability and intermediate precision [8,9]. RSD(%) for repeatability and intermediate precision did not exceed 2% (see Table 2) for the 5 components of the four levels indicating the accidental error can be accepted of the method except the least concentration level. RSD (%) for the least concentration is more than 3% means the least concentration level cannot satisfying the requirements of precision as well.

Accuracy is related to the total errors containing systematic and accidental error of the test results. Fig. (3) (1) shows that the 90% β -expectation upper and lower tolerance limits are totally included in the acceptance limits set at $\pm 10\%$ for the whole concentration range except the first concentration of ginsenoside R_1 . For notoginsenoside R_{g_1} , ginsenoside R_e , ginsenoside R_{b_1} and ginsenoside R_d , as illustrated in Fig. (3) (2)(3)(4)(5), their accuracy profiles show that the relative upper and lower 90% β -expectation tolerance limits are totally included inside the acceptance limits set at $\pm 5\%$ except the least concentration level. Consequently, the method can be considered as accurate between 0.1484 and 0.486 mg/mL for ginsenoside R_1 , between 0.558 and 2.178 mg/mL for ginsenoside R_{g_1} , between 0.104 and 0.273mg/mL for ginsenoside R_e , between 0.375 and 2.156mg/mL for ginsenoside R_{b_1} and between 0.123 and 0.306mg/mL for ginsenoside R_d (see Table 2).

Limits of quantitation (LOQ) were 0.148 mg/mL, 0.558 mg/mL, 0.104 mg/mL, 0.375 mg/mL, 0.123 mg/mL (see Table 2) of the 5 components respectively. For the LOQ it was

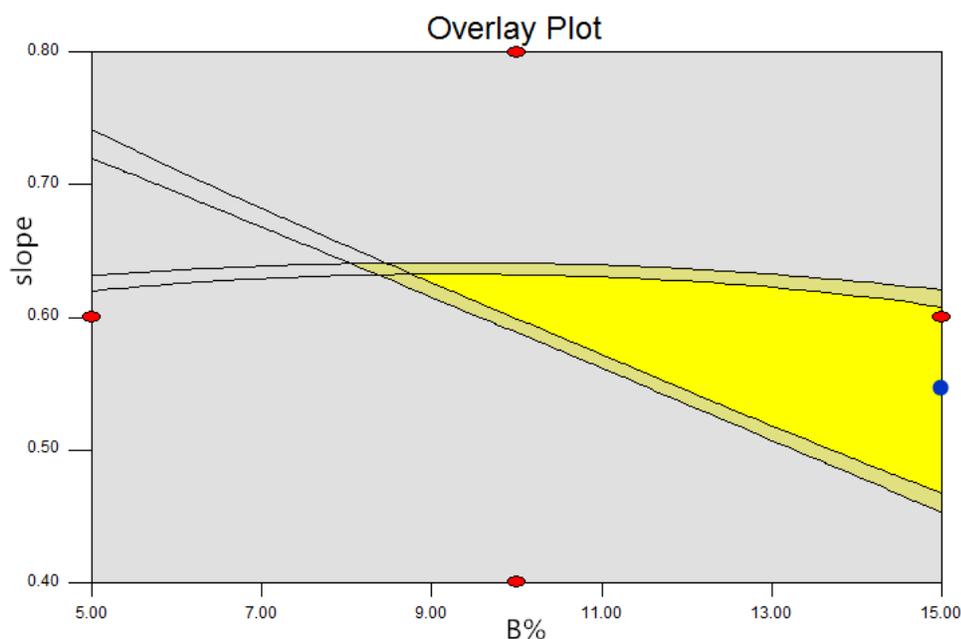


Fig. (2). The Design Space of the method. The blue dot is the optimized conditions.

Table 2. Validation results of the HPLC-UV method for the determination of the notoginsenoside Rg1 (3 series, 3 repetitions).

| | Validation parameter | | | | |
|--------------------------------|----------------------|-----------------------|---|-------------------------------|--|
| notoginsenoside R ₁ | Trueness | Concentration (mg/mL) | Relative bias (%) | | |
| | | 0.0405 | -4.374 | | |
| | | 0.203 | -3.925 | | |
| | | 0.324 | -4.840 | | |
| | | 0.486 | -3.707 | | |
| | Precision | Concentration (mg/mL) | Repeatability (RSD%) | Intermediate precision (RSD%) | |
| | | 0.0405 | 3.810 | 3.937 | |
| | | 0.203 | 0.783 | 1.231 | |
| | | 0.324 | 0.740 | 1.054 | |
| | | 0.486 | 0.996 | 0.996 | |
| | Accuracy | Concentration (mg/mL) | 90% β -expectation lower and upper tolerance limits of the relative error (%) | | |
| | | 0.0405 | [-12.23 , 3.478] | | |
| | | 0.203 | [-6.949 , -0.900] | | |
| | | 0.324 | [-7.306 , -2.374] | | |
| | | 0.486 | [-5.669 , -1.744] | | |
| | Range | 0.1484-0.4865 mg/mL | | | |
| | Linearity | Slope | 0.962 | | |
| | | Intercept | -0.0007604 | | |
| | | r | 0.9997 | | |

obtained from the accuracy profile (see Fig. 3) where the crossing point of accuracy limits and 90% β -expectation limits. The range is the interval between LOQ and the last concentration where the procedure achieves adequate accuracy.

In order to demonstrate the linearity of method, a linear regression model is fitted on the results as a function of the introduced concentrations in order to obtain linearity equation. The linearity equation of the 5 components are $Y = -0.0007604 + 0.9617X$, $Y = 0.001404 + 0.9818X$, $Y = 0.001805 + 0.9863X$, $Y = 0.004275 + 0.9905X$, $Y = -0.001496 + 1.005X$. All the r value of the 5 linearity equation are bigger than 0.9992 (see Table 2) which demonstrate the method has a good relationship between introduced and calculated concentration using β -expectation tolerance interval approach.

CONCLUSION

A novel analytical method for the separation of notoginsenoside R₁, ginsenoside Rg₁, Re, Rb₁, Rd in PNS was developed using DoE-DS methodology based on HPLC.

Interactions of the three critical parameters are showed on the design space. The method developed and validated was then successfully applied to quantify these five compounds.

Results demonstrated that the QbD approach can provide a better understanding of the critical parameters of a method and allows greater flexibility especially when time and resource are under constraints.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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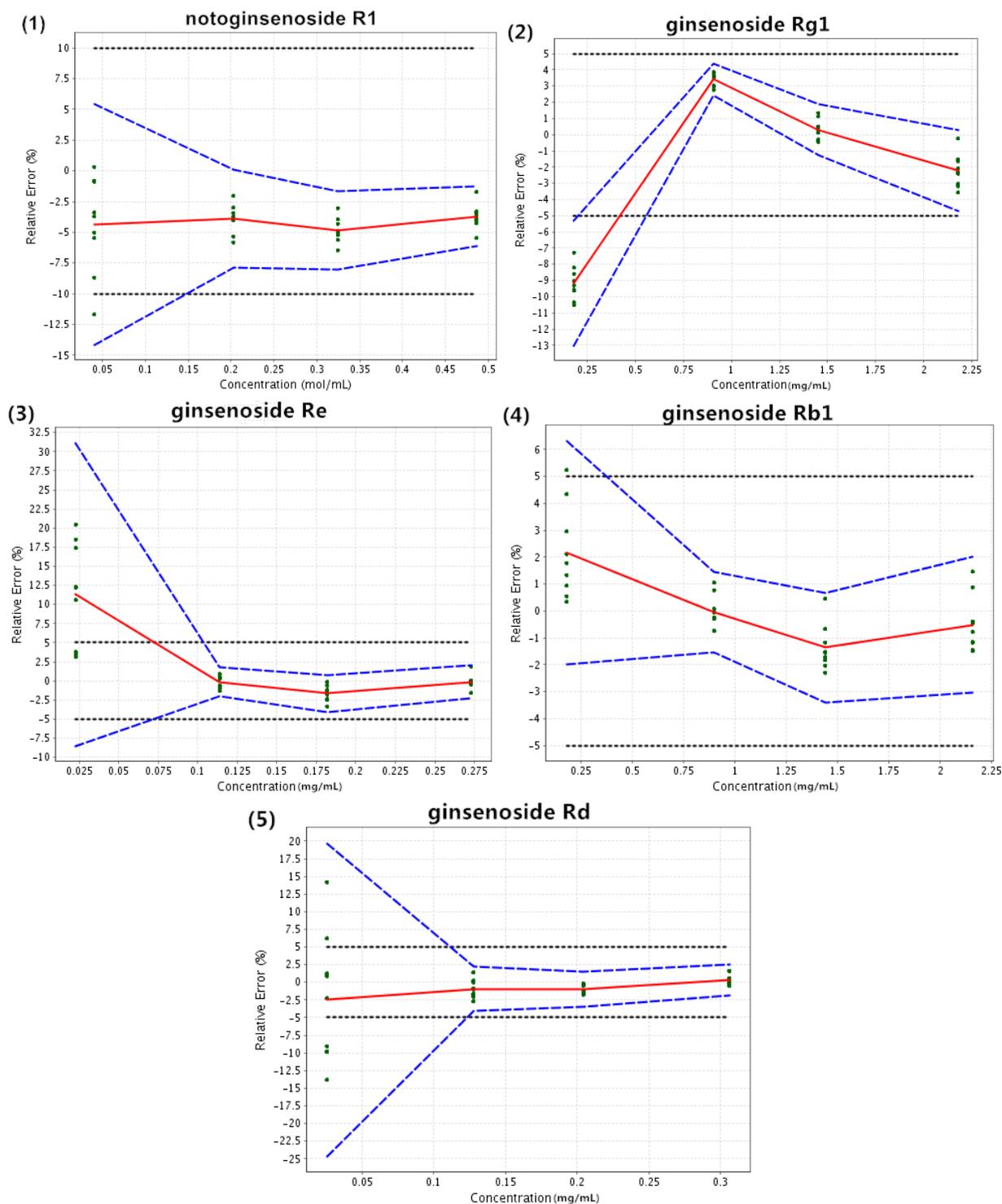


Fig. (3). Accuracy profiles of the 5 components. The plain line is the relative bias. The dashed lines are the 90% β -expectation tolerance limit and dotted lines represent the acceptance limit. The acceptance limit of the first component is $\pm 10\%$ and the rest of them are $\pm 5\%$.

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