Journal of Cytology & Histology

Shen, et al., J Cytol Histol 2014, 6:1
DOI: 10.4172/2157-7099.1000301

Research Article Open Access

Optimization and Validation of a Fingerprint about *Hypericum perforatum L.* Extracts by Plackett-Burman Randomization Method

Shu Jie Shen[1], **Yan Ru Liu**[1,2], **Chun Hua Guo**[1,3], **Rong Qing Huang**[1*], **Jian Yun Yang**[1] **and Bing Kun Xiao**[1]

[1]*Beijing Institute of Radiation Medicine, No. 27 Taiping Road, Haidian District, Beijing 100850, PR China*

[2]*Shanxi Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Shaanxi University of Chinese Medicine, No.1 Middle Weiyang Road, Qindu District, Xianyang, Shaanxi, PR China*

[3]*Jiangxi University of Traditional Chinese Medicine, No.18, Yunwan Road, Wanli District, Nanchang 330004, PR China*

[*]**Corresponding author:** Huang R-Q, Beijing Institute of Radiation Medicine, NO. 27 Taiping Road, Haidian District, Beijing 100850, PR China, Tel: 86 13681493410; E-mail: rqhuang@aliyun.com

Rec date: Nov 04, 2014, **Acc date:** Jan 07, 2015, **Pub date:** Jan 09, 2015

Abstract

Hypericum perforatum L. (HPL) has been used as a beneficial herb on menopause related syndromes for many years by inhibiting lipid oxidation. *Hypericum perforatum* L. extract contain flavonoids and phenolic acids, but it was not a method to separate and identify flavonoids which was based on design of experiment. Moreover, upper complicated HPLC method might cause undesirable effects on feasibility and repeatability in fingerprint technology. It was necessity to establish a generally and systematized way which could develop a reliable fingerprint technology for Chinese medicine. Plackett-Burman design method was a conventional tool for variables randomization aiming at optimization. Our study was based on this optimized design of experiment about ethanol extract from *Hypericum perforatum* L. and established the corresponding method for separation and identification of flavonoids. With this Design-Expert software, the results showed HCRF have significant effect on variables. From One-factor plots for main effect of HCRF, 254 nm wavelength measurement have a significant influenced on the HCRF, and have provided the optimized parameters.

Keywords: Plackett-Burman (PB) design; Flavonoids; Fingerprint technology

Introduction

Menopausal syndrome was one of the important factors, which affects the interference middle-aged and old women's life quality for many years. *Hypericum perforatum* L. has been used for the treatment of antidepressant and antioxidant widely. *Plackett-Burman* design method was a conventional tool for variables randomization. Such diversity could be within the same species. For that differentiated strains might have some minor or major differences [1,2]. This design method could enable randomizing different variables aiming to get the best conditions where each variable coordinates with other variables to give the best expected results [3]. Simple tools could be used to conduct complicated target if the correct variables have been used or if successful alternative variables are used as well. The main pharmacological activity of *Hypericum perforatum* L. was flavonoids. Flavonoids actively ingredients could remove ABTS•+(2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) on free radical) radical activity [4]. While the current study had not established the appropriate methods to separate and to identify about flavonoids on optimal design experiments (DOE, Design of Experiment) on the ethanol extract of *Hypericum perforatum* L. In addition, HPLC method might affect the feasibility and the reproducibility of fingerprints. Therefore, it is necessary to establish a reliable and easily systematic approach of *Hypericum perforatum* L. fingerprints. We choose five common and representative *Hypericum perforatum* L. flavonoids (rutin, hyperoside, quercitrin, hypericin and quercetin) based on the *Plackett-Burman* randomization method. This study was based on the optimized design of ethanol extract from *Hypericum perforatum* L. and aimed at established the corresponding method for separation and identification of flavonoids.

Materials and Methods

Materials

Standard extracts rutin (purityN97%), hyperoside (purityN98%), quercitrin (purityN98%) and quercetin (purityN98%) from HPL were obtained commercially from Must Biotechnology CO., LTD. (Chengdu, China).

Regents

Methanol (Fisher, Fair Lawn, NJ, USA) and formic acid (Fluka, Buchs, Switzerland) were of HPLC grade. The pure-distilled water used was Watsons pure distilled water.

Screening design and main effect plot

Hypericum perforatum L. and reference substance solution preparation: The standard sample of rutin, Hyperoside, quercitrin and quercetin were weighed accurately, and dissolved in methanol to prepare the solution of compounds into 0.1 mg/mL, respectively. The same operation of *Hypericum perforatum* L. from alcohol extract into 2 mg/ml.

The design of Chromatography elution system impact factor

This study was based on the formula of liquid chromatography, the elution dynamics system and the thermodynamics factors could be optimized design. In the pre-experiment, acetonitrile water system has been proved preferable results of separation condition. And specific concentrated formic acid for pH of mobile phase system has been selected as a modifier. Then other variables such as oven temperature was depend on experimental condition.

Statistical analysis

Design-Expert statistical software (Version8.0.6, Minneapolis, MN) was used to design and to analysis. In this study, the experimental P-B design (N=12) was used to identify the main factors affecting the responses among numerous variables [5]. Multifactor (such as the detection wavelength, column temperature, gradient time, sample volume, flow rate, concentration of acid, initial proportion of acetonitrile) were analysed of variance (ANOVA) after the HPLC analysis process. This experimental was paralleled operation three times. In this study, an experimental Box–Behnken response surface design was built to optimize the main factor which was affects the response among variables optimal parameters setting.

Optimization of a liquid chromatography factor

The ratio of liquid phase organic solvent was an important factor about the retention time. Due to the methanol has interacts with analyte hydrogen bond and stronger polarity than acetonitrile. So we selected acetonitrile as organic phase. Besides, column temperature was another parameter that affecting retention time of polar compound. In facts, with the increasing temperature and elevating diffusion, coefficient could generate narrow peaks and higher resolution. Taking the life-span of the column into account, the column temperature range was set in 25~40°C. So the ODS column and the mobile phase of acetonitrile-water were selected as a separate system. Meanwhile, a low concentration of formic acid was added to suppress the ionization of flavonoids and other carboxylic acids.

Plackett-Burman (PB) design of experiment

To optimize the controllable factors, including separates of column temperature, detection wavelength, injection volume, gradient elution parameters and initial concentration of organic solvent, Plackett-Burman (PB) test design method was applied, which allows the number of possible factors to be minimized with an efficiency option. Our study adopts four corresponding factors∑Rs, $r^*(x10^{-3})$and HCRF to be evaluated for the quality of the fingerprint from many aspects (as seen table 1). Besides, four corresponding factors were imported in Desigtn-Expert statistical software (Version8.0.6, Minneapolis, MN) which was based on the Plackett-Burman (PB) pilot program. It is shows that the relationship between variables and HCRF regression equation have statistically significant (p=0.0053). As a result, the equation of HCRF could evaluate the quality of fingerprints.

$HCRF=1{,}000{,}000n+100{,}00R_{min}+(t_m-t_1)$ (formula 1)

The effect on HCRF had determined with the significant p values. By the analysis of variance, learning that the statistically significant have influenced on variable factors which contains: detection wavelength, initial organic phase proportion, injection volume and velocity of flow (p=0.0005, p=0.0005, p=0.032, p=0.0444), while other factors had no effect on HCRF (P > 0.05) (Figure 1).

∑Rs	$r^*\times10^{-3}$	Φ	HCRF
21.41	129.6382	56.01743	27066001
46.52	82.33626	35.86686	10187007
39.29	147.7798	38.9847	15066009
123.36	215.547	47.86575	5483008
90.36	0.098556	467.4022	56060001
57.02	16.79956	42.18741	10160008
10.33	703.5344	49.17757	58047001
11.61	744.7321	56.39541	50043003
8.27	547.1393	52.85101	77055000
94.35	50.21223	47.18402	7154009
104.26	74.18386	73.93493	10176007
20.35	137.4934	55.45506	25051005

Table 1: Table response results.

From One-factor plots for main effect of HRCF, we found that the 254 nm wavelength measurement had effect on the HCRF distinctively. Column temperature and injection volume had elevated the spectrum of HCRF, but the longer gradient would cause the higher initial ratio organic phase. Greater acid concentration and faster flow rate were still left much to be desired in analysis.

Box-Behnken response surface analysis

The Box-Behnken response surface design was applied to find more about optimized significant factors. Hoping to find the maximum setting of HCRF, the experimental for Box-Behnken was established with four factors and three levels design.

The equation was: Ey=b0+b1z1+…+ b4z4+b11z12+…+ b11z12+ b12z1z2+…b34z3z4

From the analysis of variance, the corresponding p value =0.1098. It indicated that the equation had small proportion in the actual fitting of non-normal errors and had intimated correspond with factors. Response surface plots shows the inject volume and the detective wavelength for (a) and (b) at 254 nm and 20 ul, respectively; and the detective wavelength and initial concentration of acetonitrile for (c) and (d) at 254 nm and 15%, respectively (Figure 2).

As shown in the picture, with the same detection of wavelength, the detection wavelength had better effects on the surface than the increasing sample volume (Figure 2a and 2b). With the increasing initial organic phase, the response surface decreased gradually, but the flow velocity had no effect on it (Figure 2c and 2d). Then, the lower organic could contribute to separate polar components in *Hypericum perforatum* L. (mainly flavonoids). In other words, the detection wavelength and the initial ratio of organic phase had no interacting effect on the response factors.

With the maximum response factor from the equation, we obtain the best optimal parameters. After three repeated tests to verify the results of factor analysis, the result shows: Column: Diamonsil C18 (250 mm x 4.6 mm I.D., 5 μm.), Detection wavelength: 254 nm,

column temperature: 40°C, gradient time: 25 min, sample volume: 25 µl, Flow rate: 0.8 ml/min, the initial ratio of acetonitrile: 15%, the acid concentration: 0.05%.

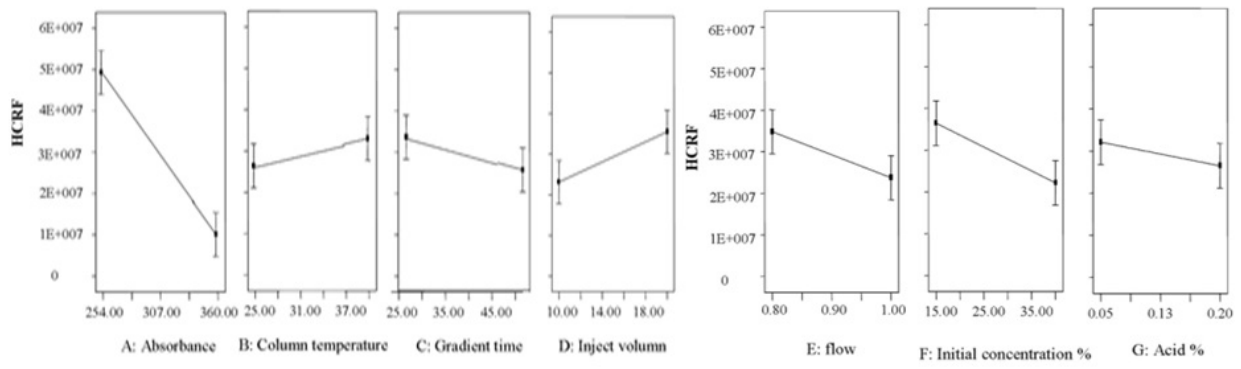

Figure 1: Response one-factor plots for main effect of HRCF, 254 nm wavelength measurement has distinctively effect on the HCRF.

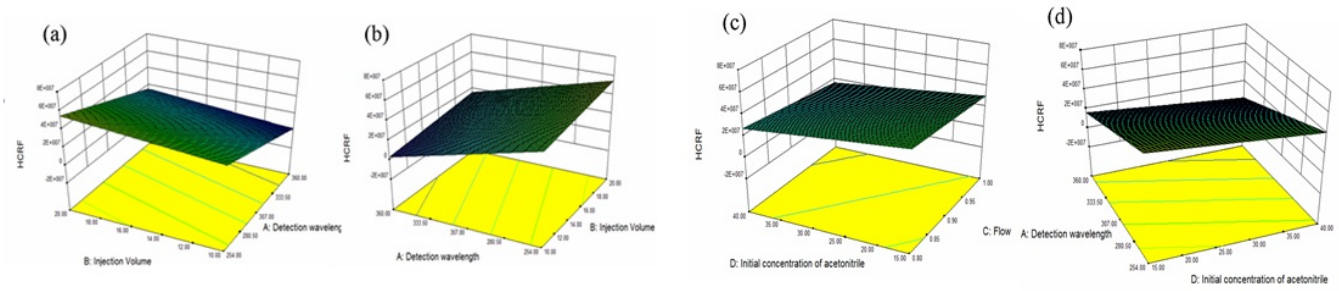

Figure 2: a, b shows that the detection wavelength had better effects on the surface than the increasing sample volume, while c, d shows that with increase in initial organic phase, the response surface decreases gradually, but the flow velocity has no effect on it.

Fingerprint similarity evaluation

Compared with standard medicinal herbs extracted from *Hypericum perforatum* L. for HPLC spectrum, and used traditional Chinese medicine (TCM) chromatographic fingerprint similarity evaluation system for the median similarity evaluation, the result shows that the average similarity was 96.0% (as seen table 2).

Compound	20121004-1	20121004-2	20121004-3	compared fingerprint(CF)
20121004-1	1			0.952
20121004-2		1		0.981
20121004-3			1	0.947
(CF)	0.952	0.981	0.947	1
Mean				0.96

Table 2: HPLC fingerprint compared between Hypericum perforatum L. extracts and standard extracts.

Method Validation

Peak identification

According to the standard chromatogram separation in the same chromatographic conditions, Main active compounds peak were identified from the fingerprint (Figure 3).

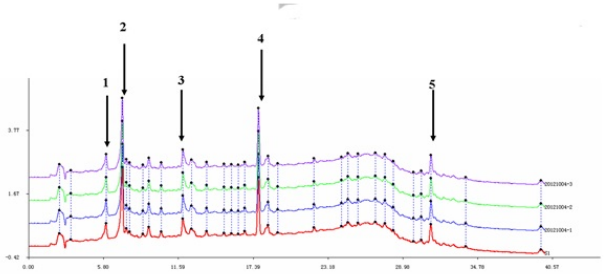

Figure 3: Compared with the standard medicinal in the same chromatographic conditions, and identified the main active compounds in the fingerprints, this *Hypericum perforatum* L. fingerprint shows: 1.rutin 2.hyperoside 3.quercitrin 4.quercetin 5.hypericin.

Linearity and range

Hyperoside standards sample (5 mg) was weighed accurately into a 25 ml brown volumetric flask. Added the mobile phase to the scale line, the stock solution was made to standard solution. Used the mobile phase concentration to dilution and 0.22 μm membrane to filter, the standard stock solution was mixed into 10~100 μg/ml linear working fluid. Besides, the working fluid was transferred to the chromatograms with 20 μl sample injection. After the test, calculation of the equation was Y=37393X+71352, r=0.9995(Figure 4).

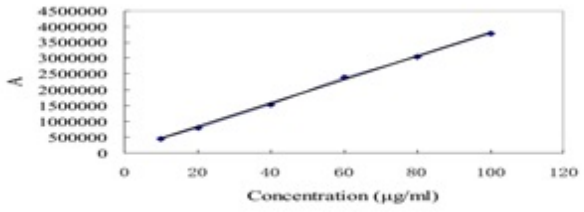

Figure 4: Linearity and range standard curves of hyperoside (10~100 μg/ml). It reveals *hyperoside* had better linearity in range from 10 μg/ml to 100 μg/ml (50%~500%LC).

Precision test

The standard stock solution of *hyperin* (20 μg/ml) were diluted with the mobile phase to prepare the concentration 80%, 100% and 120%, respectively. Each concentration had three copies and used 0.22 μm membrane to filter. Besides, the working fluid was transferred to the chromatograms with 20 μl sample injection. Calculating the method of precision and the inter day precision for each concentration. It shows that 80%, 100% and 120% solution precision were 0.52%, 0.36% and 1.28%. Meanwhile, the 72 h solution between day precision were 0.62%, 0.45% and 0.16%. Our study which established chromatographic conditions about the precision method and the inter day precision were successful.

Recovery test

According to Chinese pharmacopoeia [6] of traditional Chinese medicine (TCM) in the provisions of the quality control methods (The pharmacopoeia of the People's Republic of China. 2010), *Hypericum perforatum* L. extract powder of *hyperoside* (1 g, 6 copies) were weighed accurately into 10 ml brown volumetric flasks. *Hyperoside* standard stock solution (1 ml) was added to each copy and used the mobile phase concentration to diluting. Dissolved by the ultrasonic and using 0.22 μm membrane to filter, the solution was transferred to the chromatograms with 20 μl sample injection. Finally, the rate of *hyperin* recovery was 98~102%, RSD=1.69%, which illustrated this method had got a better accuracy

Determination of the content

Hypericum perforatum L. extract powder (5 g) were weighed accurately into the 25 ml brown volumetric flask. Besides, mobile phase was added to the scale line. Solutions which parallel to prepare three copies were dissolved by ultrasonic about 30 min and used 0.22 μm membrane to filter. What's more, the solution was transferred to the chromatograms with 20 μl sample injection. Finally, according to the external standard method, *Hypericum perforatum* L. extract powder of *hyperoside* was calculated by the peak area. The content of *hyperoside* was 0.026%, RSD=0.67%.

Conclusion

Plackett–Burman design proves to be a powerful tool for optimizing variables. The study shows 254 nm wavelength measurement had significant influence on it, and had afford better optimized parameters from One-factor plots for main effect of HCRF. In conclusion, Plackett-Burman (PB) design of experiment have established the appropriate methods to separate and to identify about flavonoids on the alcohol extract of *Hypericum perforatum* L., which could be widely used in quality control of compound extract and quality assessment process.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (Project No. 21375147), Beijing Natural Science Foundation (Project No. 7142125) and the National Key Technologies R&D Program for New Drugs of China (No. 2012ZX09301003-001-010).

References

1. Amara AA, Shibl AM, Tawfik AF (2012) Deeper in the antibiotics paradigm: microbes react individually and collectively for spreading the resistance. Afr J Microbiol Res 6: 2010–2019.
2. Amara AA (2011) Experimental design for simultaneous production of PHB, proteases and lipases. IIUM Eng J, 155–184.
3. Plackett RL, Burman JP (1946) The design of optimum multifactorial experiments. Biometrika 37: 305–325.
4. Yanru Liu, Rongqing Huang, Junxing Dong, Chenggang Zhang (2014) ABTS • + Scavenging Potency of Selected Flavonols from Hypericum perforatum L. by HPLC-ESI/MS QQQ: Reaction Observation, Adduct Characterization and Scavenging Activity Determination. Applied Food Research International, 58: 47–58.
5. Li S, Liu X, Zhu Y, Dong F, Xu J, et al. (2014) A statistical approach to determine fluxapyroxad and its three metabolites in soils, sediment and sludge based on a combination of chemometric tools and a modified

quick, easy, cheap, effective, rugged and safe method. J Chromatogr A 1358: 46-51.

6. The pharmacopoeia of the People's Republic of China (2010) ISBN 1 97-7-506-7-9-443 A national pharmacopoeia committee. Beijing, PR china.