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On-line solid-phase extraction–HPLC–fluorescence detection for simultaneous determination of puerarin and daidzein in human serum

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ABSTRACT

Response surface methodology (RSM) was applied to the optimization of on-line solid-phase extraction (SPE) parameters, and an automated system of on-line SPE coupled with high-performance liquid chromatography (HPLC) with fluorescence detection was developed for the determination of puerarin and daidzein in human serum. The human serum sample of 50 μL was injected into a conditioned C18 SPE cartridge, and the matrix was washed out with acetonitrile–KH_2PO_4–triethylamine buffer (0.01 M, pH 7.4) (3:97, v/v) for 3 min at a flow rate of 0.25 mL/min. Then the target analytes were eluted and transferred to the analytical column. A chromatographic gradient elution was programmed with the mobile phase consisting of acetonitrile and KH_2PO_4–triethylamine buffer, and the analytes were determined with a fluorescence detector at excitation wavelength of 350 nm and emission wavelength of 472 nm, respectively. The proposed method presented good linear relations (0.85–170 μg/mL for puerarin and 0.2–40 μg/mL for daidzein), satisfactory precision (RSD < 8%), and accredited recovery (92.5–107.8%).

1. Introduction

Puerarin and daidzein (Fig. 1) are two main active isoflavones of *Radix Puerariae* [1,2], an important crude herb. Pharmacologic and clinical studies have shown that puerarin could be used to treat hypertension, angina pectoris, and acute myocardial infarction [3]; and that daidzein has an effect on anoxia, cerebral ischemia [4,5] and angiocardiopathy [6]. In order to investigate the pharmacokinetic (PK) properties of puerarin and daidzein, it is urgently needed to develop a simple and effective method for the quantitative analysis of them in biomatrices. Many attempts have been made so far in analyzing puerarin in *R. Puerariae* and its medicinal preparations with high-performance liquid chromatography [7–11]. Several methods have also been employed to determinate puerarin and other bioactive constituents, including capillary zone electrophoresis (CZE) with a UV detector [12,13], and HPLC-UV [14]. However, the sample preparation is crucial for the analysis of biological sample because of the extremely complex interference in biomatrix. The solid-phase extraction (SPE) technique has been widely used to remove the interfering biomatrix substances [15–17], and reported for the analysis of puerarin in plasma [18]. The off-line manual procedure was relatively tedious, time consuming, and sample-consuming. Therefore, lots of researches focused on the automated on-line SPE system coupled with HPLC [19–26].

The coupling technique has such advantages as reducing time and solvent volume used, and avoiding many of the problems associated with more traditional approaches. The efficiency of SPE is determined by many parameters and their interactions. Zivanovi et al. has applied the fractional factorial design and the central composite design to the optimization of SPE for mycophenolic acid and mycophenolic acid glucuronide [27]. The response surface methodology (RSM) could be constructed for exploring the approximate functional relationship between the response variable and the set design variables [28], and has been used to explain the combined effect of the factors in the extraction process [29–31]. However, to our knowledge, no research has applied RSM to the optimization of SPE procedure.

The objective of this study was to establish an efficient and automated method of on-line SPE coupled with HPLC for the determination of puerarin and daidzein in human serum, and to explore the possibility of optimization and development of SPE procedure with RSM.

2. Experiment

2.1. Instrumentation

HPLC analysis was done on a Waters Breeze liquid chromatography system (Waters Corporation, Milford, MA, USA),

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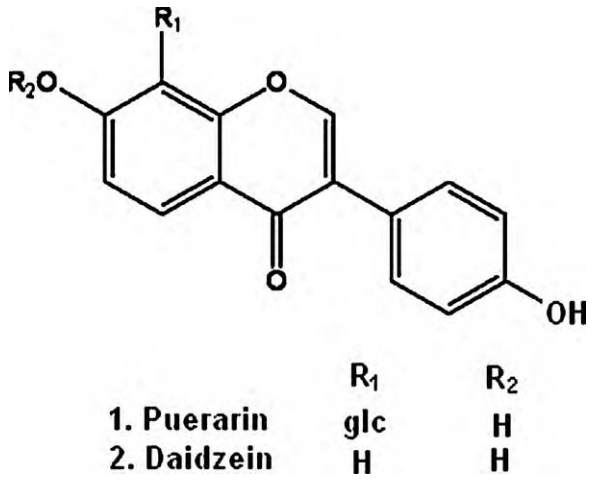

Fig. 1. Structures of puerarin and daidzein.

which comprises 1525 binary high-pressure pump, 7725i injection valves and a thermostatted column compartment controlled with Empower Workgroup. A Waters 2996 diode-array detector recorded the absorption spectra of analytes, and a Waters 2475 multi-wavelength fluorescence detector was used for the quantitative detection. Chromatographic separations were carried out on an Agilent HC-C18 column (250 mm × 4.6 mm; i.d., 5.0 μm, Agilent Corporation, Wilmington, DE, USA). The solid-phase extraction system composed of an EasySep-1010 LC high-pressure pump (Tongwei Biological Science & Technology Co., Ltd., Shanghai, China), a Waters 7725i injection valves with a sample loop of 50 μL, and an Agilent C18 guard column (4.6 mm i.d. × 20 mm) used as the solid-phase extraction cartridge.

2.2. Materials and chemicals

The standard compounds of puerarin and daidzein were obtained from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Methanol and acetonitrile (HPLC grade) were obtained from Honeywell International Inc. (Muskegon, MI, USA). The ultra-pure water was prepared with Milli-Q system (Bedford, MA, USA). The human serum samples were obtained from Shaanxi Normal University Hospital.

2.3. Chromatographic condition

Acetonitrile (A) and KH_2PO_4–triethylamine buffer (0.01 M, pH 7.4) (B) were used as the mobile phase. The gradient elution programed was: 0–25 min, 15–40% A; 25–30 min, 40–15% A. The flow rate of mobile phase was set at 0.8 mL/min. The column temperature was maintained at 30 °C. The absorption spectra were recorded ranging from 220 nm to 400 nm. The fluorescence excitation and emission wavelengths were 350 and 472 nm (18 nm bandwidth), respectively.

2.4. Preparation of standard solutions

Puerarin (5.1 mg) and daidzein (1.2 mg) were accurately weighed and placed in a 10-mL volumetric flask. The methanol solution (30%) was used as the solvent for preparing the stock solution of 510 mg/L puerarin and 120 mg/L daidzein, respectively. The appropriate quantity of stock solutions were taken and diluted with 30% aqueous methanol to make a series of mixed standard solutions (2.55, 5.1, 25.5, 51, 102, 255 and 510 μg/mL for puerarin; and 0.6, 1.2, 6.0, 12, 24, 60 and 120 μg/mL for daidzein).

2.5. Preparation of serum sample and serum calibration standard solution

The real serum samples were taken from the patients under therapy with Fenghan Ganmao Keli, a Chinese traditional medicine containing puerarin and daidzein, at Shaanxi Normal University Hospital. Two hours after the first daily drug administration, the blood samples were collected and then treated into serum. The drug-free serum samples were also obtained from Shaanxi Normal University Hospital. Serum calibration standard solutions (0.85, 1.7, 8.5, 17, 34, 85 and 170 μg/mL for puerarin; and 0.2, 0.4, 2, 4, 8, 20 and 40 μg/mL for daidzein) were obtained by adding 100 μL mixed standard solution to 200 μL blank serum, respectively.

2.6. Extraction procedure for on-line analysis

The SPE–HPLC system setup and the operating processes are shown in Fig. 2. The extraction procedure consisted of three processes: sampling, solid-phase extraction, and elution.

In the sampling process (A), the serum sample was injected into the 50 μL sample loop, and Pumps 1 and 2 were both activated simultaneously. Pump 1 was used for the conditioning of SPE with the mixture of acetonitrile and KH_2PO_4–triethylamine buffer (v/v, 3/97) at the flow rate of 0.25 mL/min. Pump 2 (binary pump) was used for establishing the baseline of the detector with initial mobile phase (15% acetonitrile).

In the solid-phase extraction process (B), the sampling valve was turned to the injection position. The sample in the loop was loaded into SPE cartridge and the matrix was washed out with the carrier of acetonitrile/KH_2PO_4–triethylamine buffer for 3 min.

Finally, the valve with SPE cartridge was turned to the injection position (C). The analytes trapped on SPE cartridge were eluted into the analytical column by gradient elution.

2.7. Design of RSM for optimization of extraction parameters

A three-variable and three-level of Box–Behnken design (BBD) [28], one method of the RSM, was adopted to optimize the extraction procedure. The independent variables were chosen as organic solvent of different polarity (*S*, mixt with buffer at a ratio of 3 to 97, v/v), pH value of buffer (pH), and the flow rate of SPE pump (*V*, at a fixed loading and washing time of 3 min). Three levels of each variable were coded as −1, 0, and +1 (Table 1). The chromatographic peak area of puerarin was taken as response, *Y*. A regression analysis was performed for the experimental data to fit into an empirical second-order polynomial model.

3. Results and discussion

3.1. Optimization of solid-phase extraction parameters

At on-line SPE, the trapped efficiency of analytes on column and the cleared content of matrix depend entirely on the carrier for the given cartridges. The SPE parameters optimized include the composition, pH, and the flow rate of carrier and the washing time. The composition of carrier was considered as the organic solvent of different polarity (methanol, acetonitrile and ethanol) and its ratio, and the kind of buffer (KH_2PO_4–NaOH, Na_2HPO_3–KH_2PO_4, Tris–HCl and KH_2PO_4–triethylamine) and its concentration. Based on the results of a series of one-variable-at-a-time experiments, KH_2PO_4–triethylamine buffer of 0.01 M and organic solvent of 3% were determined. The other factors effecting extraction efficiency were further optimized with the RSM.

The empirical second-order polynomial model obtained with RSM for the SPE of puerarin was established as follows:

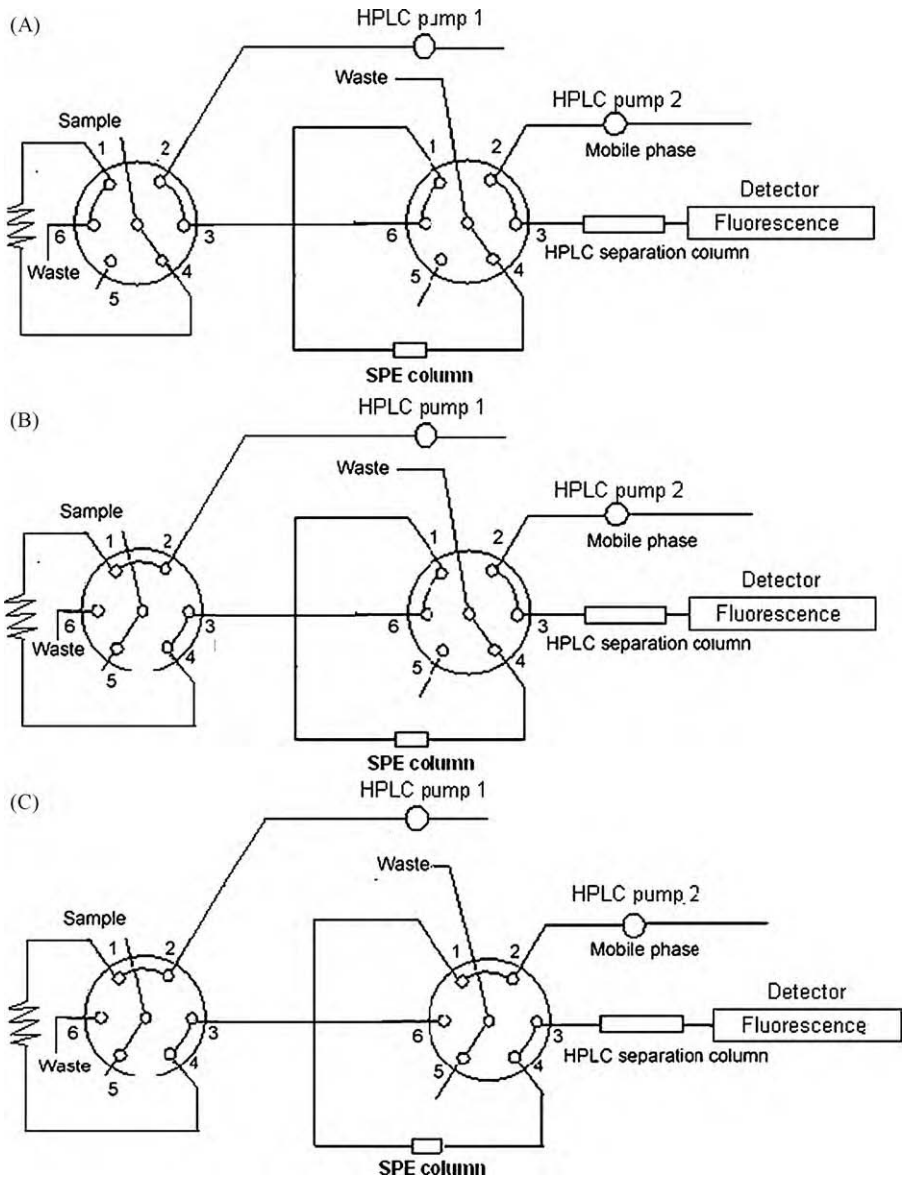

Fig. 2. Manifold diagram of SPE–HPLC–FD system. A: Sampling; B: Solid-phase extraction; C: Elution and HPLC analysis.

Table 1
BBD experiment design and result for the optimization of on-line SPE.

Run	Factor 1, *S*	Factor 2, *V* (mL/min)	Factor 3, pH	Response	
				Observed peak area	Predicted peak area
1	Methanol (−1)[a]	0.1 (−1)	7.5 (0)	655245	757650
2	Ethanol (1)	0.5 (1)	7.5 (0)	803010	700600
3	Acetonitrile (0)	0.1 (−1)	6 (−1)	821835	798000
4	Methanol (−1)	0.3 (0)	9 (1)	725821	756700
5	Acetonitrile (0)	0.5 (1)	6 (−1)	770270	903500
6	Methanol (−1)	0.5 (1)	7.5 (0)	834510	779800
7	Acetonitrile (0)	0.3 (0)	7.5 (0)	939880	939400
8	Ethanol (1)	0.3 (0)	9 (1)	453792	532300
9	Methanol (−1)	0.3 (0)	6 (−1)	774330	695800
10	Acetonitrile (0)	0.3 (0)	7.5 (0)	934820	939400
11	Ethanol (1)	0.1 (−1)	7.5 (0)	564150	618900
12	Acetonitrile (0)	0.1 (−1)	9 (1)	930320	797000
13	Acetonitrile (0)	0.5 (1)	9 (1)	771545	795400
14	Ethanol (1)	0.3 (0)	6 (−1)	733230	702300
15	Acetonitrile (0)	0.3 (0)	7.5 (0)	943496	939400

[a] Numbers in brackets are the coded values of the independent variables in the experimental design.

$$Y = 9.20 \times 10^5 - 1.18 \times 10^4 S - 1.58 \times 10^4 V - 2.25 \times 10^3 \mathrm{pH} - 1.58 \times 10^4 SV - 5.99 \times 10^4 S\mathrm{pH} + 3.09 \times 10^4 V\mathrm{pH} - 1.40 \times 10^5 S^2 - 2.61 \times 10^4 V^2 - 1.10 \times 10^5 \mathrm{pH}^2,$$

where Y represented the response, chromatographic peak area of puerarin; and S, pH and V correspond to three independent variables, organic solvent, pH value of the buffer, and the flow rate of carrier at a fixed loading and washing time of 3 min, respectively.

An analysis of variance (ANOVA) was performed to determine the significance (Table 2). The coefficient of determination (R^2) of 0.9480 for model predicted that the model has adequately represented the real relationship between the parameters chosen. The model F value of 10.12 implied that the model is significant. There is only a 1.01% chance that the model could occur due to noise. A non-significant lack of fit ($p > 0.05$) showed that the quadratic model is valid to the spatial influence of variables on the response. Furthermore, the interaction between organic solvent and pH value of the buffer, and the quadratic terms of organic solvent and buffer pH value have statistically significant effects on SPE of puerarin ($p < 0.05$).

Three-dimensional (3D) surface plots and contour plots were constructed as shown in Fig. 3. The 3D surface plots showed visually the effects and interaction of two independent variables on the responding variable as third independent variable was fixed at the central experimental level of zero. It could be seen from Fig. 3 that organic solvent and buffer pH value were more important factors effecting SPE efficiency than the flow rate of carrier, and that all the optimal parameters were nearly at the central experimental level of zero.

From the result of RSM, the optimal SPE procedure was as follows: taking the mixture (3:97, v/v) of acetonitrile and KH_2PO_4–triethylamine buffer (0.01 M, pH 7.4) as the carrier, loading the serum sample (50 μL) into SPE cartridge and washing the matrix for 3 min at a flow rate of 0.25 mL/min.

3.2. Optimization of the chromatographic system

The chromatographic system was optimized in order to match with the fluorescence detector. Puerarin and daidzein have similar fluorescence spectra, excitation wavelengths at about 260 and 350 nm, respectively, and a maximum emission wavelength at 472 nm. Compared with 260 nm, stronger fluorescence intensity was observed at the excitation wavelength of 350 nm. Therefore, 350 and 472 nm were selected as excitation and emission wavelengths for HPLC detection, respectively.

Gradient elution is widely applied to improve mixture separation in analytic liquid chromatography. In the present work, the optimal gradient was ascertained through many attempts. With a gradient elution of acetonitrile and KH_2PO_4–triethylamine buffer solution (0.01 M, pH 7.4) on Agilent HC-C18 column, a good separation was achieved within 30 min with the retention times of 7.9 ± 0.1 min for Puerarin and 21.7 ± 0.2 min for Daidzein, respectively. The typical chromatograms for standard and serum sample were shown in Fig. 4. Almost no interference was present in the chromatographic separation, and each target peak had a good resolution.

3.3. Method validation

3.3.1. Specificity

The interference of endogenous compounds in the serum was assessed by comparing the chromatograms of standard puerarin and daidzein, drug-free serum, serum spiked with puerarin and daidzein, and serum samples obtained from the patients treated with Fenghan Ganmao Keli. Because the high selectivity of fluorescence detection, all peaks shown in the chromatograms were essentially free from the endogenous interferences. The retention times were 7.9 ± 0.1 min for puerarin and 21.7 ± 0.2 min for daidzein, and the peak shapes were satisfactory and suitable.

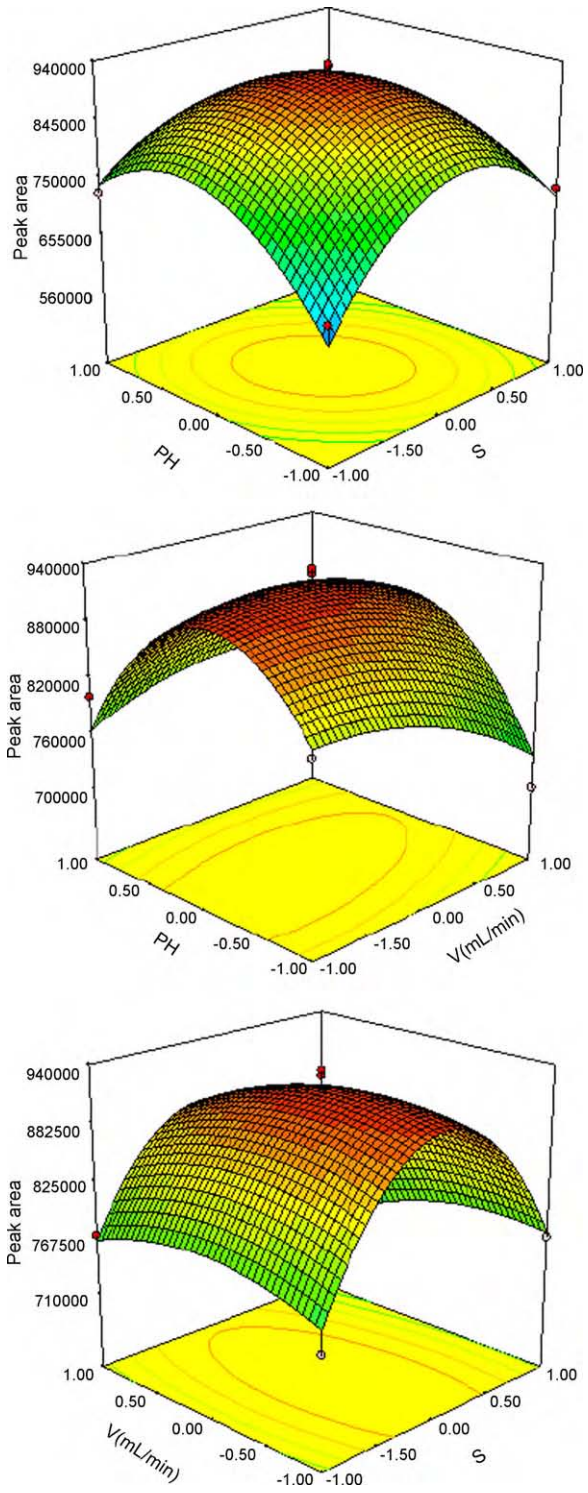

Fig. 3. Response surface plots for the effects of parameters on on-line SPE of puerarin. A: the pH value of buffer solution and different organic solvent (S); B: the pH value of buffer solution and the flow rate of carrier (V); C: the flow rate of carrier (V) and different organic solvent (S).

3.3.2. Linearity

The linearity of the calibration curve for puerarin and daidzein were assessed with six serum standard solutions. The regres-

Table 2
Analysis of variance (ANOVA) for the quadratic polynomial model.

Source[a]	Sum of squares	DF[b]	Mean square	*F* value	*p* value
Model	1.309E+011	9	1.454E+010	10.12	0.0101[c]
S	1.116E+009	1	1.116E+009	0.78	0.4185
V	1.992E+009	1	1.992E+009	1.39	0.2919
pH	4.066E+007	1	4.066E+007	0.028	0.8730
SV	1.001E+009	1	1.001E+009	0.7	0.4419
*S*pH	1.436E+010	1	1.436E+010	10.00	0.0251[c]
*V*pH	3.813E+009	1	3.813E+009	2.65	0.1642
S^2	7.040E+010	1	7.040E+010	49.01	0.0009[c]
V^2	2.519E+009	1	2.519E+009	1.75	0.2427
pH^2	4.598E+010	1	4.598E+010	32.01	0.0024[c]
Lack of fit	6.174E+009	3	2.058E+009	4.08	0.2031
Pure error	1.009E+009	2	5.046E+008		
R^2	0.9480	Adjusted R^2	0.8543		
CV (%)	4.91	PRESS	1.011E+011		

[a] Coefficients refer to the general model.
[b] Degree of freedom.
[c] $p < 0.05$.

Table 3
Method precision of SPE–HPLC–FD ($n = 6$).

Analyte	Concentration (μg/mL)	Intra-day			Inter-day		
		Found	RSD (%)	Recovery (%)	Found	RSD (%)	Recovery (%)
Puerarin	8.5	8.6 ± 0.2	2.3	101.2	8.4 ± 0.4	5.4	98.8
	34.0	36.8 ± 2.4	6.5	108.2	35.6 ± 2.1	6.0	104.7
	85.0	82.5 ± 4.7	5.7	97.1	88.3 ± 0.5	5.7	103.9
Daidzein	2.0	2.1 ± 0.1	6.7	105.0	1.9 ± 0.1	6.3	95.0
	8.0	7.7 ± 0.4	5.2	96.2	8.5 ± 0.6	7.5	106.2
	20.0	19.7 ± 0.6	3.0	98.5	21.1 ± 1.1	5.1	105.5

Table 4
Analytical results and recoveries for puerarin in human serum samples ($n = 6$).

Sample	Content (μg/mL)	Spiked (μg/mL)	Found (μg/mL)	Recovery (%)
Patient 1	0	8.5	8.7 ± 0.3	102.4
		34.0	33.1 ± 2.3	97.4
Patient 2	11.4 ± 0.6	8.5	20.5 ± 1.6	107.0
		34.0	47.4 ± 3.1	105.9
Patient 3	19.9 ± 0.8	8.5	28.3 ± 2.4	98.8
		34.0	55.5 ± 3.5	106.2

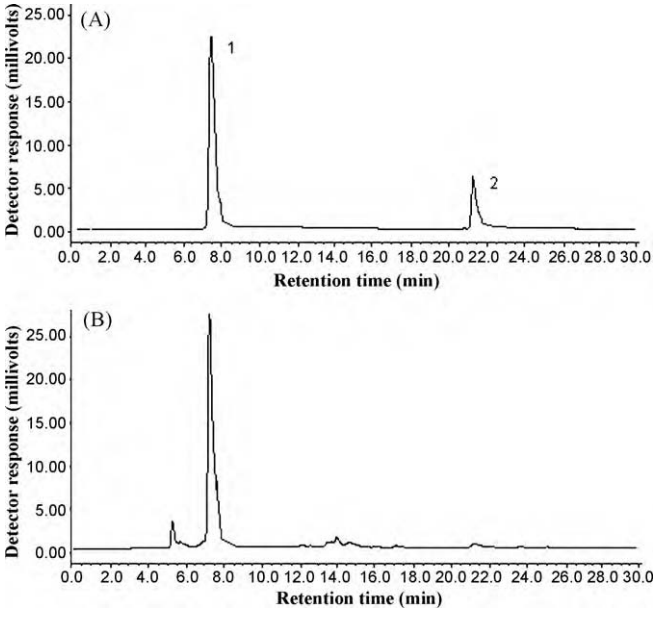

Fig. 4. HPLC–FD chromatograms. A: for the standard solution, 8.5 μg/mL of puerarin (1) and 2 μg/mL of daidzein (2); B: for the serum sample.

sion equation, peak area (Y) against the concentration (X, μg/mL), was derived as $Y = 16705X + 408977$ ($R^2 = 0.9975$) for puerarin; and $Y = 18830X + 9311$ ($R^2 = 0.9994$) for daidzein. The linear ranges were 0.85–170 and 0.2–40 μg/mL, respectively. The limit of detection (LOD), defined as the lowest analyte concentration which could be detected (S/N > 3), was 0.06 μg/mL for puerarin, and 0.03 μg/mL for daidzein. The limit of quantification (LOQ) calculated (S/N > 10) was 0.21 μg/mL for puerarin, and 0.11 μg/mL for daidzein.

3.3.3. Precision and ruggedness

The precision of the method was obtained by calculating the relative standard deviation (RSD) from repeated injections of the serum standard solutions at 8.5, 34 and 85 μg/mL for puerarin and 2, 8 and 20 μg/mL for daidzein. The intra-day precision was determined by six replicate injections, while the inter-day precision was determined by six injections for 6 continuous days. The intra-day precision of less than 7% and inter-day precision of less than 8% were obtained (Table 3).

The comparable results of series of spiked serum samples on the same HPLC system indicated the ruggedness of proposed method.

3.4. Application to real patient serum samples

The proposed method has been applied to determination of puerarin and daidzein in the real patient serum samples, and the

Table 5
Analytical results and recoveries for daidzein in human serum samples ($n = 6$).

Sample	Content (μg/mL)	Spiked (μg/mL)	Found (μg/mL)	Recovery (%)
Patient 1	0	0.4	0.37 ± 0.05	92.5
		2	2.12 ± 0.17	106.0
Patient 2	0.54 ± 0.10	0.4	0.91 ± 0.10	92.5
		2	2.45 ± 0.23	95.5
Patient 3	0.86 ± 0.12	0.4	1.29 ± 0.15	107.5
		2	2.94 ± 0.16	104.0

results are showed in Tables 4 and 5. The samples were spiked with standard compound of the analytes in two different amounts. Recoveries were obtained by comparing the amounts of analytes added to the sample with the amounts detected. The recoveries from 92.5% to 107.8% were obtained for the two analytes. The results showed that the proposed method was simple, sensitive, selective and reliable for the determination of puerarin and daidzein in human serum.

4. Conclusion

Response surface methodology provides an impactful way to establish the optimal conditions for on-line solid-phase extraction of drug from biological matrices with a limited number of experiments. Combining the rapid and efficient on-line drug SPE with the specific and sensitive HPLC–fluorescence detection, an accurate and reproducible SPE–HPLC–FD method for determination of puerarin and daidzein in human serum has been developed and validated. The analytical results demonstrated that the proposed method is suitable for the quantification of puerarin and daidzein in human serum without interferences from the endogenous compounds and co-administered drugs. The performances of simplicity, reliability and ruggedness make it valuable in the routine therapeutic monitoring and pharmacokinetic studies of puerarin and daidzein.

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