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Optimization of on-line solid phase extraction and HPLC conditions using response surface methodology for determination of WM-5 in mouse plasma and its application to pharmacokinetic study

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ABSTRACT

Response surface methodology (RSM) was utilized for rapid and systematic optimization of on-line solid-phase extraction (SPE) parameters to maximize the response and separation of WM-5. The optimization was performed with Box–Behnken designs. Four major parameters were investigated for their contributions to the response and separation of WM-5, with a total of 29 experiments being performed for each instrument, respectively. Quantitative determination of WM-5 in mouse plasma was performed to evaluate the statistical significance of the parameters on chromatographic response. A fully automated on-line SPE and high-performance liquid chromatography (HPLC) with diode array detection (DAD) method was developed for the determination of WM-5 in mouse plasma. Calibration curve with good linearity ($r = 0.9989$) was obtained in the range of 20–4000 ng/mL in mouse plasma. The limit of detection (LOD) and lower limit of quantification (LLOQ) of the assay were 6 ng/mL and 20 ng/mL, respectively. The overall intra-day and the inter-day variations were less than 1.90%. The recovery of the method was in the range of 93.74–96.33% with RSD less than 3.06%. The optimized method demonstrated good performance in terms of specificity, LLOQ, linearity, recovery, precision and accuracy, and was successfully applied to quantify WM-5 in mouse plasma to support the pharmacokinetic study.

1. Introduction

Phenanthroindolizidine alkaloids are plant-derived alkaloids widely presented in the *Asclepiadaceae*, *Moraceae*, *Acanthaceae* and *Lauraceae* plant families [1]. Chemical investigations and pharmacological studies have demonstrated that phenanthroindolizidine alkaloids have a series of biological and physiological activities, such as anti-cancer [2], anti-inflammatory [3], anti-anaphylactic [4], anti-leukemia [5], anti-asthma [6], etc. Increasing attention has been attracted since the first phenanthroindolizidine alkaloid-tylophorine isolated and identified with its unique biological activity from *Asclepiadaceae* plant family [7,2,8]. WM-5 ((*S*)-2,3-dimethoxy-9,11,12,13,13a,14-hexahydrodibenzo[*f*,*h*]pyrrolo[1,2-*b*]isoquinolin-6-ol) (Fig. 1a) is a synthetic derivative of phenanthroindolizidine alkaloids isolated from *Cynanchum komarovii* Al. Iljinski, discovered as an anti-cancer agent by Prof. Qingmin Wang at Nankai University. It showed excellent potency with median effect concentration (EC_{50}) less than 0.3 nM against human lung adenocarcinoma A-549, significantly better than that of commercialized anti-cancer drugs such as paclitaxel, methotrexate, vincristine, cisplatin and adriamycin [9]. WM-5 is currently undergoing intensive preclinical profiling including *in vivo* efficacy, safety and developability, etc. to investigate its potential as an anti-cancer drug candidate. As a critical part of the preclinical profiling, pharmacokinetic (PK) study could not only link data from pharmacological assays to therapeutic applications, but also help design rational dosage regimens and minimize adverse effects. However, to our knowledge, PK study of WM-5 has not yet been reported. It is of great value to develop an efficient and reliable bioanalytical protocol for determination of WM-5 in biological matrix with hopefully maximum response and isolation, and to study the PK properties of Wm-5 to advance its preclinical development.

Conventional PK study involves a complex manual pretreatment process to eliminate the complex sample matrix (precipitation, liquid–liquid extraction, membrane filtration, off-line SPE, etc.), which has its drawbacks such as time-consuming, labor intensive,

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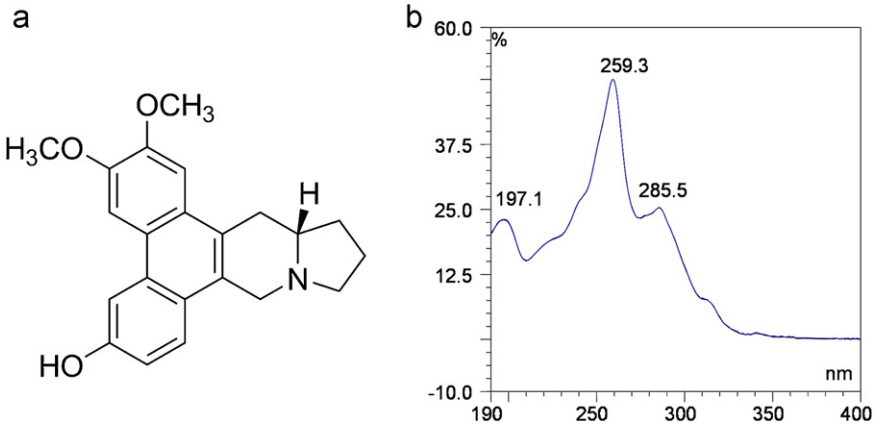

Fig. 1. (a) Chemical structure and (b) characteristic UV absorption wavelength of WM-5.

error-prone and costly [10–15]. Optimization of the clean-up of complex matrix such as plasma and urine, with aim to enable high sample throughput, achieve total automated procedure, reduce the cost and improve overall analytical quality, has attracted considerable attention. Automated on-line SPE is becoming a plausible solution which integrating the biological fluids clean-up process with analysis [16–20]. Comparing with the conventional HPLC system, the automated on-line column-switching HPLC system shows significant advantages as follows: first of all, nearly no pretreatment procedure of complex matrix samples to fulfill the increasing demand of bioanalysis, and higher throughput with superior sensitivity, accuracy, precision and recovery since nearly no matrix effect or carry over could be observed with this method, while it could not be achieved by the conventional HPLC methods since the possible missing and error-prone brought by the manual pretreatment process. Moreover, relative lower cost of the analytical equipments and the consumables. The last but not the least, less volume of sample can achieve more scientific results than that of conventional HPLC methods since possible hypovolemia and dehydration altering the PK parameters are avoided, also less exposure to hazard solvents ensures the safety of this methodology [21–23].

A typical on-line SPE procedure is generally carried out as follows: after a direct injection of PK plasma sample into the instrumentation, the analytes are trapped on the SPE cartridge while the matrix components being flushed to waste. By rotation of the switching valve, the analytes are eluted and transferred to the analytical column and quantified consequently. The success of on-line SPE is determined by many parameters and their interactions. For instance, the optimization of chromatographic conditions and on-line SPE procedure such as the correct valve switching times, and creation of two alternative control programs require advanced knowledge of the analytical and applied data systems. However, relative investigation of those parameters in on-line SPE was rare or not systematic, even though a number of on-line SPE–HPLC bioanalytical protocols have been published [24,25].

Response surface methodology (RSM) has been utilized to study the relative relationship between the chromatographic response and the set design variables, and explain the combined effect of the factors in the extraction and separation process [26,27]. RSM has been applied as optimization approach for many liquid chromatographic studies, and also considered as the most appropriate solution for building response surface to predict the optimized chromatographic conditions [28–31]. Compared with orthogonal design, this multivariate approach could acquire more precise and useful information by conducting a minimal number of experiments and evaluating the set design variables through performed regression equations [32–34]. This paper describes a multivariate optimization approach using response surface techniques to optimize on-line SPE process for the development of an efficient, reliable and fully automated on-line solid phase extraction and high-performance liquid chromatography conditions for determination of WM-5 in mouse plasma with hopefully maximum sensitivity and resolution. Method validation in terms of calibration curves, extraction recovery, limit of detection (LOD), lower limit of quantification (LLOQ), precision, accuracy and stability is reported. The application of the established automated on-line SPE–HPLC-DAD enabling the efficient and reliable PK study of WM-5 in mice is presented.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were purchased from Tedia (Fairfield, Ohio, USA). Ultra pure water was produced from a Milli-Q50 SP Reagent Water System (Bedford, MA, USA). Other reagents were of analytical grade or higher if not otherwise stated. WM-5 was kindly supplied by Prof. Qingming Wang (State Key Laboratory of Elemento-Organic Chemistry, Nankai University), the structure was identified by spectroscopic methods (UV, IR, MS, ^{1}H NMR and ^{13}C NMR), and the purity was determined to be over 98% by HPLC.

2.2. Preparation of calibration standards and quality control samples

The stock solution of WM-5 was prepared by dissolving requisite amount in acetonitrile at a high concentration (1 mg/mL), and then diluted with acetonitrile/H_2O (50/50 v/v) for further concentration series of 40,000 ng/mL, 10,000 ng/mL, 5000 ng/mL, 2000 ng/mL, 1000 ng/mL, 500 ng/mL and 200 ng/mL. Calibration work solutions were prepared by adding the diluted stock solutions into blank mouse plasma (10/90 v/v) to give final concentration series of 4000 ng/mL, 1000 ng/mL, 500 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL and 20 ng/mL. Quality control (QC) samples including low (40 ng/mL), middle (400 ng/mL) and high (2000 ng/mL) concentrations of standards were also prepared as the same manner. All solutions were stored at 4 °C until HPLC analysis.

2.3. Preparation of dosing solution

Appropriate amount of WM-5 was dissolved in polyethylene glycol 400/H_2O (40/60) to obtain a clear solution, then diluted to final concentration of 5 mg/mL for both per os (PO) and intravenous (IV) administration. The dosing solution for IV administration was

filtered through a 0.22 μm filter from millipore (Molsheim, Alsace, France).

2.4. Instrumentation

HPLC analysis was carried out on a Dionex (Sunnyvale, CA, USA) Ultimate 3000 HPLC system equipped with a SRD-3600 degasser, a DGP-3000SD pump, a WSP-3000SL analytical autosampler, a TCC-3000SD column compartment and a DAD-3000 diode array detector. Data acquisition and calculation were performed using chromeleon software from Dionex (Sunnyvale, CA, USA). The chromatographic separation was performed on a Venusil MP C18 column (5 μm, 4.6 mm × 150 mm, Bonna-agla Technologies) with the column temperature set at 30 °C. The mobile phase consisted of acetonitrile-5 mM NaH_2PO_4 buffer (26/74 v/v) (pH 3.6, adjusted by 0.1 M phosphoric acid solution) at a flow rate of 1 mL/min. The DAD detector recorded UV spectra in the range from 190 to 400 nm and the monitored wavelength was set at 260 nm since the optimal absorption exhibited under this condition (Fig. 1b). The injection volume was 10 μL. A Universal 320R-refrigerated centrifuge equipped with a swing out rotor (12-place, 5000 rpm, Cat. No. 1628A) from Hettich (Kirchlengern, Germany) was employed in the plasma sample preparation.

2.5. On-line SPE–HPLC-DAD

An on-line SPE methodology was applied to pre-treat the sample, by using a SPE cartridge MF Ph-1 (10 mm × 4 mm, 5 μm) from Shiseido (Tokyo, Japan). 5 mM NaH_2PO_4 buffer (pH 3.6) was the washing solvent.

The system setup for on-line SPE was constructed with three steps (Fig. 2). In the first step (loading), 10 μL of plasma sample was loaded onto the SPE cartridge using an Ultimate 3000 RS autosampler. The SPE cartridge was fitted into loading position of Valco 6-port switching valve. The loading pump (right pump) was used to load the plasma sample onto the SPE cartridge, and the biological matrix was flushed to waste for 1 min with the washing solvent (5 mM NaH_2PO_4 buffer, pH 3.6) at a flow rate of 1 mL/min, while WM-5 was retained on the stationary phase of the SPE cartridge and simultaneously the analytical column was equilibrated with the chromatographic pump (left pump). In the second step (injection), the Valco 6-port switching valve was switched to injection position that coupled the SPE cartridge with the analytical column, in which WM-5 was eluted from the SPE cartridge in the back-flush mode and transferred to the analytical column for 1 min by the chromatographic mobile phase consisted of acetonitrile-5 mM NaH_2PO_4 buffer 26/74 (v/v, pH 3.6) at a flow rate of 1 mL/min. In the last step (separation), the Valco 6-port switching valve was switched back to the loading position that made the SPE cartridge and analytical column in parallel. WM-5 was separated in the analytical column for 6 min with the chromatographic mobile phase consisted of acetonitrile-5 mM NaH_2PO_4 buffer 26/74 (v/v, pH 3.6) at a flow rate of 1 mL/min. The on-line SPE cartridge was washed with acetonitrile-5 mM NaH_2PO_4 buffer 90/10 (v/v, pH 3.6) for 2 min during the separation step and then re-equilibrated with 5 mM NaH_2PO_4 buffer as initial condition for analyzing the next sample (Fig. 2). The duration of the on-line SPE loading step and chromatographic separation of WM-5 was 8 min.

2.6. Design of RSM for optimization of extraction parameters

RSM with a four-variable and three-level of Box–Behnken design (BBD) was employed to optimize the extraction procedure. The independent variables were chosen as matrix depletion time (t(M)), transfer time (t(T)), pH value of NaH_2PO_4 buffer (pH), and the flow rate of washing solvent (F). Three levels of each variable were set as −1, 0, and +1 (Tables 1 and 2). The three-dimensional response surface plots were obtained by Design-Expert® 8.0.6 software (Minneapolis, MN, USA). All experiments were performed in triplicate and the average chromatographic peak area of WM-5 was taken as response. A quadratic multiterm regression equation was performed for the experimental data to fit into an empirical second-order polynomial model.

Table 1
Factors and levels of response surface methodology test.

Factors	Coded	Level		
		−1	0	1
Flow rate of washing solvent (mL/min)	F	0.5	1	1.5
Matrix depletion time (min)	t(M)	0.5	1	1.5
pH value of NaH_2PO_4 buffer	pH	2	3.5	5
Transfer time (min)	t(T)	0.5	1	1.5

2.7. Method validation

The method was validated for linearity, LOD, LLOQ, accuracy and precision, extraction recovery and stability following the industrial guidelines of bioanalytical method validation from the U.S. Food and Drug Administration (FDA) [35].

2.8. Plasma PK studies of WM-5 in mice

Healthy Kunming (KM) male mice (body weight: 22–25 g) were obtained from Laboratory Animal Center, Academy of Military Medical Science (Beijing, China). The animals were maintained in propylene cages (target conditions: temperature 20 to 25 °C, relative humidity 40–70% and 12 h dark-light cycle) with free access to standard laboratory food and water for 5 days acclimation. Mice in oral dosing group were fasted the night before (12 h prior to the experiment), no food was allowed until 4 h post dose while water intake was free. The mice were divided into two dose groups

Table 2
Results of response surface methodology test.

Experiment No.	Level				Peak area (mAU*min)
	F	t(M)	pH	t(T)	
1	0	0	1	−1	3.0471
2	0	0	0	0	4.1323
3	1	0	0	−1	3.1354
4	−1	1	0	0	3.2624
5	0	0	1	1	2.3764
6	−1	−1	0	0	3.3369
7	0	1	−1	0	2.2838
8	0	−1	1	0	2.5162
9	0	0	−1	1	2.7649
10	−1	0	1	0	2.3489
11	0	0	0	0	4.2365
12	0	−1	0	1	3.5616
13	1	−1	0	0	2.9384
14	0	0	0	0	4.0104
15	1	0	1	0	2.6523
16	0	0	0	0	4.3453
17	0	−1	−1	0	2.4362
18	0	0	−1	−1	2.7417
19	0	1	0	−1	3.6927
20	1	0	0	1	3.3964
21	−1	0	0	−1	3.0421
22	1	0	−1	0	2.3723
23	0	1	0	1	3.3241
24	−1	0	−1	0	2.3972
25	0	0	0	0	4.2861
26	1	1	0	0	3.1728
27	0	−1	0	−1	3.4103
28	−1	0	0	1	3.0531
29	0	1	1	0	2.7971

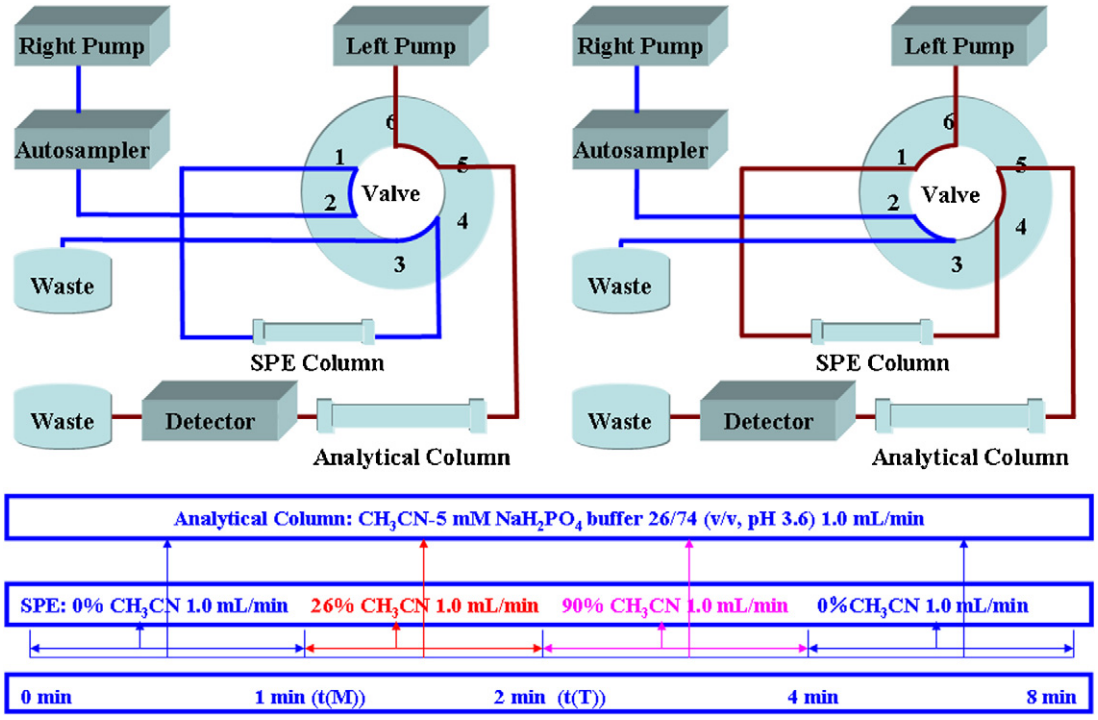

Fig. 2. The schematic diagram of on-line SPE–HPLC-DAD system.

randomly, 84 mice in each group, 6 mice were bled per time point. The mice in group 1 were dosed with WM-5 by gavage at 200 mg/kg, while in group 2 were dosed via caudal vein at 40 mg/kg. Venous blood (200 μL) was obtained from the orbit vein and collected in heparin pretreated polypropylene centrifuge tubes at 0 min, 2 min (IV only), 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 1.5 h (PO only), 2 h, 4 h, 6 h, 8 h, 12 h and 24 h (PO only) post-dose. All blood samples were immediately centrifuged to collect plasma samples. The plasma samples were stored frozen in a freezer set to maintain −20 °C until bioanalysis. The animal facilities and protocols were approved by the Institutional Animal Care and Use Committee of Nankai University. All procedures were carried out in accordance with the Guidelines of Animal Experimentation of Nankai University (Tianjin, China).

The plasma concentrations of WM-5 were determined by using established optimal on-line SPE–HPLC-DAD method with optimal conditions. PK parameters were carried out by non compartmental methods using WinNonlin version 5.2 from Pharsight Corporation (Sunnyvale, CA, USA), including highest observed plasma concentration (C_{max}), time when highest plasma concentration observed (T_{max}), mean residence time (MRT), terminal or elimination half-life ($T_{1/2}$), area under the plasma concentration–time curve from Time 0 to the last measured concentration (AUC_{0-t}), and area under the plasma concentration–time curve extrapolated to infinity (AUC_{0-INF}). In addition, the total body clearance (CL) and steady-state volume of distribution (V_{ss}) were calculated after IV dosing, together with an estimate of absolute bioavailability (F).

3. Results and discussion

3.1. Optimization of solid-phase extraction parameters

In on-line SPE process, the trapping efficiency of WM-5 on column and the clearance of endogenous matrix depended primarily on the given SPE cartridge. The key SPE parameters were SPE cartridges selection, the composition and flow rate of washing solvent (F), matrix depletion time (t(M)), pH value of washing solvents (pH), and transfer time (t(T)). Based on the results of a series of one-variable-at-a-time experiments, MF Ph-1 cartridge was chosen in the present study for its robustness and long lifetime with high protein content samples and high aqueous mobile phase, particularly allowing back flush model in the injection step. The life of the SPE cartridge was ~300 injections with 10 μL plasma sample per injection. Different washing solvents (methanol–water, acetonitrile–water, phosphates buffer, methanol–phosphates buffer and acetonitrile–phosphates buffer) were examined. Acetonitrile–NaH_2PO_4 buffer of 5 mM gave the most satisfied extraction of WM-5, since it not only gave the fitted pH (pH 3.6) value that avoided precipitation in the SPE cartridge, but also was fully compatible with SPE cartridge requirement as indicated by the manufacturer. Consequently, the other key factors were optimized with a multivariate approach using RSM.

The empirical second-order polynomial model obtained with RSM for the SPE of WM-5 was established as following:

$$\begin{aligned} Y = {} & 4.20212 + 0.01892F + 0.02777t(\mathrm{M}) + 0.06183\mathrm{pH} \\ & - 0.04940t(\mathrm{T}) - 0.63171F^2 - 0.42868t(\mathrm{M})^2 - 1.17848\mathrm{pH}^2 \\ & - 0.32701t(\mathrm{T})^2 + 0.07723Ft(\mathrm{M}) + 0.08207F\mathrm{pH} \\ & + 0.06250Ft(\mathrm{T}) + 0.10832t(\mathrm{M})\mathrm{pH} - 0.12997t(\mathrm{M})t(\mathrm{T}) \\ & - 0.17348\mathrm{pH}t(\mathrm{T}) \end{aligned}$$

where Y represented the response, chromatographic peak area of WM-5; and F, t(M), pH and t(T) correspond to four independent variables, the flow rate of washing solvent, matrix depletion time, pH value of NaH_2PO_4 buffer and transfer time, respectively.

The regression coefficients and the corresponding P-values were shown in Table 3. As can be seen from Table 3, the regression coefficients of the quadratic terms of pH^2, F^2, $t(M)^2$ and $t(T)^2$ were significant. An analysis of variance (ANOVA) was employed to determine the significance (Table 4). The results of ANOVA implied that this regression model was highly significant ($P < 0.01$) with F-value of 23.77. A non-significant lack of fit ($P = 0.240$) indicated that the quadratic model was valid to the spatial influence of variables

Table 3
The central composite parameter estimation of four levels and three factors.

Variables	Coefficient	Coefficient standard error	*T*	*P*
Intercept	4.20212	0.08005	52.496	0.000
F	0.01892	0.05167	0.366	0.720
t(M)	0.02777	0.05167	0.538	0.599
pH	0.06183	0.05167	1.197	0.251
t(T)	−0.04940	0.05167	−0.956	0.355
F^2	−0.63171	0.07028	−8.989	0.000
$t(M)^2$	−0.42868	0.07028	−6.100	0.000
pH^2	−1.17848	0.07028	−16.769	0.000
$t(T)^2$	−0.32701	0.07028	−4.653	0.000
Ft(M)	0.07723	0.08949	0.863	0.403
*F*pH	0.08207	0.08949	0.917	0.375
Ft(T)	0.06250	0.08949	0.698	0.496
t(M)pH	0.10832	0.08949	1.210	0.246
t(M)*t*(T)	−0.12997	0.08949	−1.452	0.168
pH*t*(T)	−0.17348	0.08949	−1.938	0.073

on the response. Also it was further confirmed by a satisfactory value of determination coefficient (R^2) of 0.9596, which indicated that 95.96% of the variability in the response could be predicted by the model.

Three-dimensional (3D) surface plots and contour plots were constructed as shown in Fig. 3. The 3D surface plots visualized the effects and interaction of two independent variables on the response while other independent variables were fixed at the central experimental level of zero. According to the degree of affecting the peak area of WM-5, the order of the four parameters was listed as: pH > *t*(T) > *t*(M) > *F*.

RSM optimization led to the optimal on-line SPE process as following: taking NaH_2PO_4 buffer (5 mM, pH 3.6) as the washing solvent, loading the plasma sample into SPE cartridge and washing for 1 min at a flow rate of 1 mL/min. By rotation of the switching valve, the WM-5 was eluted and transferred to the analytical column and separated for 1 min, the Valco 6-port switching valve was then switched back to the loading position for analyzing the next sample.

3.2. Optimization of the chromatographic system

Liquid chromatographic conditions including stationary phase, the composition and pH value of mobile phase, column temperature and flow rate were investigated. Venusil MP C18 column was chosen in the present study for its high efficiency and peak symmetry. Different mobile phases (methanol–water, acetonitrile–water, methanol–acid aqueous solution, acetonitrile–acid aqueous solution, methanol–buffer, and acetonitrile–buffer) were examined to obtain efficient chromatography and relatively short run time for WM-5. It was found that addition of acetonitrile could remarkably improve the peak shape of WM-5. In the meantime, the washing solvent of MF Ph-1 cartridge is limited to only phosphates buffer as recommended by the manufacturer. Therefore, the mobile phase was selected as acetonitrile–NaH_2PO_4 buffer (5 mM, pH 3.6) to achieve better separation and less interference from other components in the plasma. The best separation and optimum analytical speed were found at a condition of isocratic elution at a flow rate of 1 mL/min while column temperature being kept at 30 °C.

3.3. Method validation

3.3.1. Specificity and selectivity

The method specificity and selectivity was tested by using visual inspectional of the chromatograms of blank plasma from six mice, blank plasma spiked with WM-5, and plasma samples obtained from PK studies. Fig. 4 shows the chromatograms of the above-mentioned samples. No interference from endogenous materials or other sources was observed at the retention time of the analyte 6.46 min in blank plasma.

3.3.2. Linearity, LOD and LLOQ

The method linearity was assessed using the WM-5 plasma standards over a concentration range of 20–4000 ng/mL. Each calibration curve was individually fitting the area response of WM-5, using least square weighted ($1/x^2$) linear regression and excluding the point of origin. Nice linearity (Linear regression equation: $y = 0.2526x + 0.0002$, *x* and *y* represent the relative concentration and the peak area of WM-5, respectively) and good coefficients of determination ($r = 0.9989$) were obtained. The LOD was determined by successive analysis of mouse plasma with decreasing amounts of WM-5 until a signal-to-noise ratio (S/N) being 3 reached, and the value of LOD was calculated as 6 ng/mL. The LLOQ samples from six different mouse plasma independent of the calibration curves were analyzed. A signal-to-noise ratio (S/N) at 10 was observed for WM-5. The LLOQ of the assay was determined to be 20 ng/mL.

Table 4
Variance analysis of regression equation.

Source	Degree of freedom (DF)	Seq SS	Adj SS	Adj MS	*F*	*P*	Determination coefficient (R^2)
Regression	14	10.6619	10.6619	0.76156	23.77	0.000	0.9596
Linear	4	0.0887	0.0887	0.02218	0.69	0.610	
Square	4	10.2719	10.2719	2.56796	80.16	0.000	
Interaction	6	0.3013	0.3013	0.05022	1.57	0.228	
Residual error	14	0.4485	0.4485	0.03204			
Lack-of-fit test	10	0.3782	0.3782	0.03782	2.15	0.240	
Pure error	4	0.0704	0.0704	0.01759			
Total	28	11.1104					

Table 5
Intra-day and inter-day precisions and accuracies of the assays ($n = 5$).

Nominal concentration (ng/mL)	Intra-day Concentration found (ng/mL)		RSD (%)	Accuracy (%)	Inter-day Concentration found (ng/mL)		RSD (%)	Accuracy (%)
	Mean	SD			Mean	SD		
40.00	39.47	0.31	0.79	98.68	39.24	1.15	2.93	98.10
400.00	395.52	2.28	0.58	98.88	393.55	4.28	1.09	98.39
2000.00	1987.36	8.94	0.45	99.37	1976.93	23.44	1.19	98.85

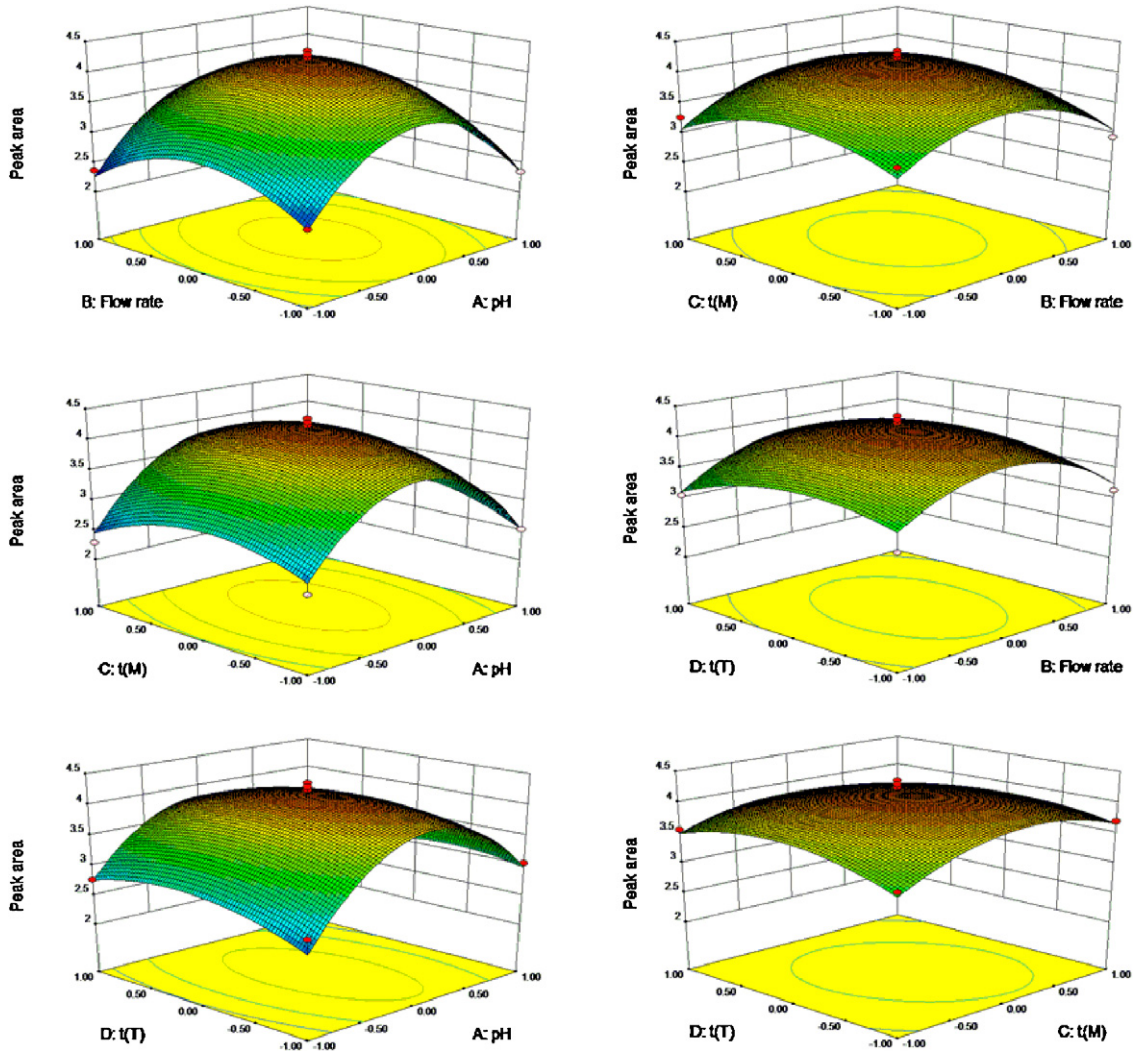

Fig. 3. Response surface plots for the effects of parameters on on-line SPE of WM-5.

3.3.3. Accuracy and precision

The intra-day accuracy and precision were assessed by measuring QC samples at three concentration levels in five replicates, while the inter-day accuracy and precision were evaluated using single QC sample at three concentration levels on five consecutive validation days. The accuracy was expressed as percentages of nominal values. The precision was calculated as relative standard deviation (RSD) using ANOVA with day as the grouping variable. The RSD of QC samples were in the range of 0.45–2.93% and the accuracies were between 98.10% and 99.37% (Table 5), which were all within the defined acceptance criteria of USFDA.

3.3.4. Extraction recovery

The extraction recovery of WM-5 from plasma was tested by comparing the absolute response of five replicate QC samples at three concentration levels (40, 400 and 2000 ng/mL) to which the analyte at equivalent concentrations in mobile phase and expressed in percentage. The extraction recovery ranged from 93.74% to 96.33% with RSD value between 2.67% and 3.06% ($n = 5$), which indicated that the method was consistent, precise and reproducible (Table 6).

3.3.5. Stability

The stability of WM-5 in mouse plasma was tested by comparing the response of freshly prepared QC samples with that maintained at the storage conditions to be measured (Three cycles of freezing at −20 °C and thawing for freeze and thaw stability, 24 h at room temperatures for short-term temperature stability, and 4 weeks at −20 °C for long-term stability). No significant difference of concentrations for WM-5 in mouse plasma was observed during the 24 h period of assay. The deviation between the initial concentrations and the concentrations of WM-5 stored at −20 °C for 4 weeks was less than 8.02% (Table 7). The concentrations of QC samples were not significantly affected by the freezing and thawing test. After completion of three freezing and thawing cycles, the recoveries of WM-5 were between 95.17% and 97.03%.

3.4. Sample analysis and PK studies

In the present study, WM-5 could be monitored in plasma for up to 12 h post-dose after single oral administration at 200 mg/kg

Table 6
The extraction recovery of WM-5 from mouse plasma ($n = 5$).

Concentration spiked (ng/mL)	Extraction recovery (%)		RSD (%)
	Mean	SD	
40.01	93.74	2.87	3.06
400.14	95.26	2.54	2.67
2000.23	96.33	2.63	2.73

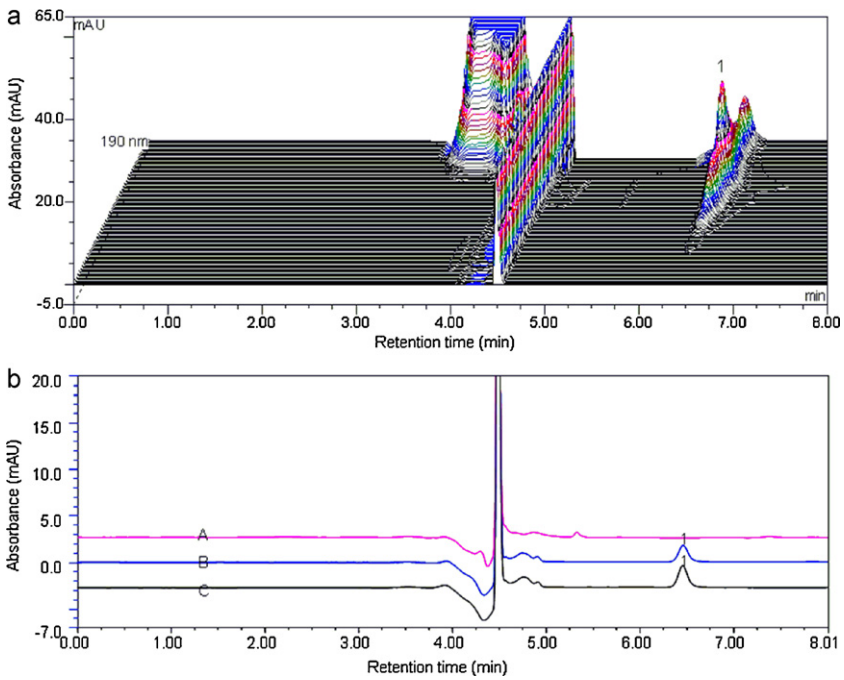

Fig. 4. (a) Three-dimensional chromatogram of blank plasma spiked with WM-5 and (b) HPLC chromatograms of (A) blank plasma, (B) blank plasma spiked with WM-5, (C) plasma sample of PO administrated mice at time point of 2 h (1 WM-5).

Table 7
Stability results of WM-5 in spiked plasma samples (n= 5).

Freshly prepared concentration (ng/mL)	Short-term stability (24 h at room temperature)			Long-term stability (4 weeks at −20 °C)			Freeze-thaw stability (3 cycles)		
	Concentration found (ng/mL)		Deviation (%)	Concentration found (ng/mL)		Deviation (%)	Concentration found (ng/mL)		Deviation (%)
	Mean	SD		Mean	SD		Mean	SD	
40.03	39.57	1.64	1.15	36.82	1.28	8.02	38.59	1.17	3.60
400.11	392.44	9.82	1.92	371.38	8.64	7.18	388.24	8.25	2.97
2000.25	1983.53	53.26	0.84	1922.45	56.83	3.89	1903.63	51.46	4.83

to male KM mice. The mean plasma concentration–time profiles were shown in Fig. 5, and the PK parameters of WM-5 were presented in Table 8. Following IV administration of WM-5 at 40 mg/kg, clearance was moderate (Mean CL = 40.75 mL/min/kg) that corresponding with approximately 45.28% of hepatic blood flow. The mean volume of distribution of WM-5 was 5374.23 mL/kg, which was 7.41 times of total body water volume (725 mL/kg) [36], indicating high extravascular distribution. The mean terminal half-life following IV dosing was 6.31 h, which was reflected a tight correlation between the clearance and terminal half-life. Following single oral administration of WM-5 solution at 200 mg/kg, maximum mean plasma concentrations were

Table 8
Absolute bioavailability and PK parameters of WM-5 following single oral and intravenous administration to male KM mice ($n = 6$).

PK Parameters	Intravenous (40 mg/kg)		Oral (200 mg/kg)	
	Mean	SD	Mean	SD
C_{max} (ng/mL)	37,472.83	6630.61	21,366.95	7429.01
T_{max} (h)	0.037	0.02	0.17	0.00
AUC_{0-t} (ng/mL h)	17,052.23	4680.09	23,667.12	9548.98
AUC_{0-INF} (ng/mL h)	17,345.77	4754.74	23,912.99	9596.13
$T_{½}$ (h)	6.31	2.48	1.89	0.15
MRT (h)	2.25	0.39	1.98	0.13
CL (mL/min/kg)	40.75	10.18	–	–
V_{ss} (mL/kg)	5374.23	989.78	–	–
F (%)	100	–	27.76	–

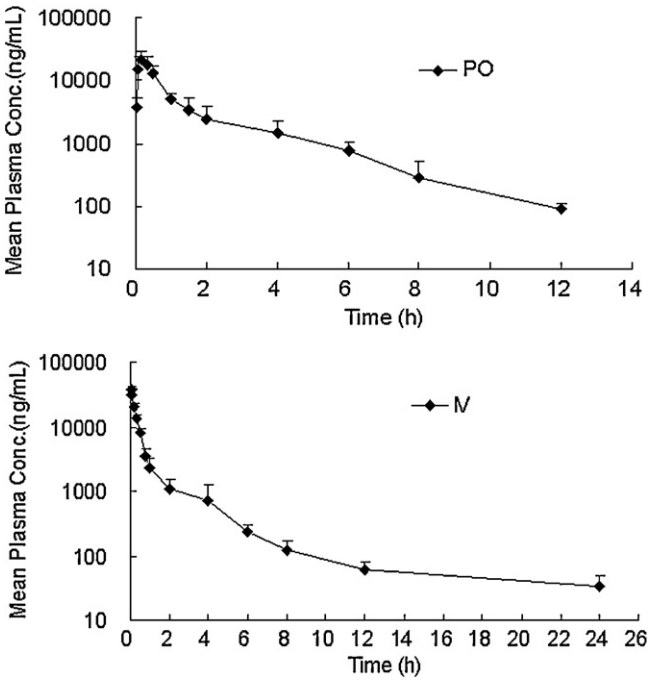

Fig. 5. Plasma concentration–time profiles of WM-5 following single oral and intravenous administration to male KM mice ($n = 6$).

exhibited as 21,366.95 ng/mL observed at approximately 0.17 h post dose (T_{max}). The rapid oral absorption would be presumably due to its low polar characteristic and small molecular size. The mean observed bioavailability of WM-5 was 27.76%. The results properly indicated that extensive intestinal first-pass elimination or high extravascular distribution would be exhibited by WM-5 *in vivo*.

4. Conclusion

Response surface methodology was employed to establish the optimal conditions for on-line SPE of WM-5 from biological matrices with a limited number of experiments. Combining the rapid and efficient on-line SPE with the specific and sensitive HPLC-DAD, an efficient, accurate and reproducible on-line SPE–HPLC-DAD method for determination of WM-5 in mouse plasma has been developed and validated with a single run in 8 min. The analytical results demonstrated good performance in terms of linearity, specificity, detection and quantification limits, precision and accuracy, and was successfully utilized to quantify WM-5 in mouse plasma to support the PK studies.

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