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Development of a fully automated on-line solid phase extraction and high-performance liquid chromatography with diode array detection method for the pharmacokinetic evaluation of bavachinin: A study on absolute bioavailability and dose proportionality

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ABSTRACT

A fully automated on-line solid-phase extraction (SPE) and high-performance liquid chromatography (HPLC) with diode array detection (DAD) method was developed for determination of bavachinin in mouse plasma. Analytical process was performed on two reversed-phase columns (SPE cartridge and analytical column) connected via a Valco 6-port switching valve. Plasma samples (10 μL) were injected directly onto a C18 SPE cartridge (MF Ph-1 C18, 10 mm × 4 mm, 5 μm) and the biological matrix was washed out for 2 min with the loading solvent (5 mM NaH_2PO_4 buffer, pH 3.5) at a flow rate of 1 mL/min. By rotation of the switching valve, bavachinin was eluted from the SPE cartridge in the back-flush mode and transferred to the analytical column (Venusil MP C18, 4.6 mm × 150 mm, 5 μm) by the chromatographic mobile phase consisted of acetonitrile-5 mM NaH_2PO_4 buffer 65/35 (v/v, pH 3.5) at a flow rate of 1 mL/min. The complete cycle of the on-line SPE purification and chromatographic separation of the analyte was 13 min with UV detection performed at 236 nm. Calibration curve with good linearity ($r = 0.9997$) was obtained in the range of 20–4000 ng/mL in mouse plasma. The intra-day and inter-day precisions (RSD) of bavachinin were in the range of 0.20–2.32% and the accuracies were between 98.47% and 102.95%. The lower limit of quantification (LLOQ) of the assay was 20 ng/mL. In conclusion, the established automated on-line SPE-HPLC-DAD method demonstrated good performance in terms of linearity, specificity, detection and quantification limits, precision and accuracy, and was successfully utilized to quantify bavachinin in mouse plasma to support the pharmacokinetic (PK) studies. The PK properties of bavachinin were characterized as rapid oral absorption, high clearance, and poor absolute bioavailability.

1. Introduction

Psoralea corylifolia, which derived from dried ripe fruit of *P. corylifolia* L., is widely used in treatment of asthma, diarrhea, vitiligo, alopecia areata, enuresis, pollakiuria, waist and knee psychroalgia, kidney weak, bone fracture, osteomalacia and osteoporosis [1–6]. As one of the most essential bioactive components in *P. corylifolia*, bavachinin (shown in Fig. 1a) exhibited a wide range of biological activities, such as antioxidant [7–9], antibacterial [10], antifungal [11,12], antiinflammatory [13,14], antitumor [15], antipyretic and analgesic properties [16]. Although with so many beneficial activities, no report of the pharmacokinetic (PK) study of bavachinin could be found in literature survey. Therefore, it is of great importance to establish a fast, specific and sensitive method to determine bavachinin in biological fluids to evaluate the potential for its therapeutic application.

A large number of bio-analytical methods based on high-performance liquid chromatography (HPLC) technology have been successfully developed for the determination of drug concentration in biological fluids for decades. However, conventional HPLC methods for the PK study of drugs in biological fluids have been proved to be time-consuming, labor intensive, error-prone and costly, primarily caused by the complex manual pretreatment steps to eliminate the complex sample matrix (precipitation, liquid–liquid extraction, membrane filtration, off-line SPE, etc.) [17–22]. In order to tackle the obstacle of sample pretreatment and accelerate analysis in PK and other matrix based biological studies, different approaches for sample pretreatment and chromatographic development have been proposed and investigated. Among which, optimization of the clean-up of complex matrix such as plasma and urine, with aim to enable high sample throughput, achieve total

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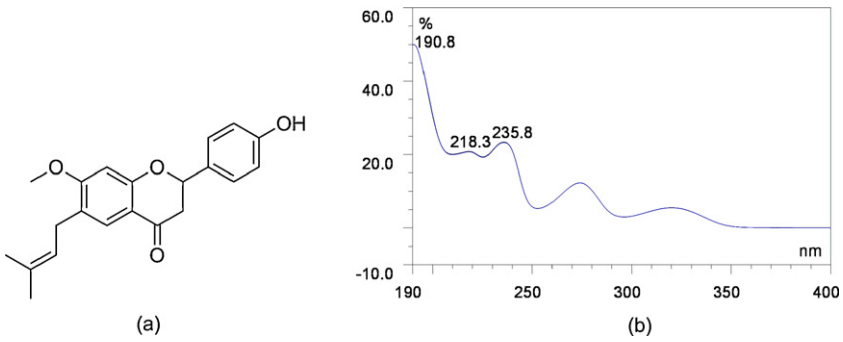

Fig. 1. (a) Chemical structure and (b) characteristic UV absorption wavelength of bavachinin.

automated procedure, reduce the cost and improve overall analytical quality, has attracted considerable attention. In this paper, we report a fully automated on-line SPE-HPLC-DAD method which integrating the biological fluids clean-up process with bio-analysis. After a direct injection into the instrumentation, the analytes were trapped on the SPE cartridge while the matrix components were flushed to waste. By rotation of the switching valve, the analytes were eluted and transferred to the analytical column and quantified consequently. This method was systematically optimized for determination of bavachinin in mouse plasma for maximum of sensitivity and resolution. Method validation in terms of calibration curves, extraction recovery, lower limit of quantification (LLOQ), precision, accuracy and stability was described.

2. Materials and methods

2.1. Chemicals and reagents

Bavachinin was kindly supplied by Prof. Zhinan Yin (College of Life Science, Nankai University). Acetonitrile and ethanol of HPLC grade were obtained from Tedia (Fairfield, OH, USA). A Milli-Q50 SP Reagent Water System used to prepare ultrapure water was from Millipore (Bedford, MA, USA). Other reagents were of analytical grade or higher if not otherwise stated.

2.2. Preparation of calibration standards and quality control samples

Bavachinin was dissolved in dimethyl sulfoxide (1.0 mg/mL) as the stock solution, 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), 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 were obtained. 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 procedure as the calibration standards. Calibration work solutions and QC samples were stored at −20 °C until HPLC analysis.

2.3. Preparation of dosing solution

Appropriate amount of bavachinin was dissolved in polyethylene glycol 400/ethanol/H_2O (60/10/30) to give a clear solution, then diluted to final concentration series of 1 mg/mL, 2 mg/mL and 4 mg/mL for per os (PO). For intravenous (IV) administration, the dosing solution (5 mg/mL) was filtered through a 0.22 μm filter from Millipore (Molsheim, Alsace, France).

2.4. Instrumentation

On-line SPE and HPLC analysis was carried out using a Dionex UltiMate 3000 HPLC system (Sunnyvale, CA, USA) equipped with UltiMate 3000 pump, UltiMate 3000 autosampler column compartment and UltiMate 3000 photodiode array detector. Data acquisition was performed with chromeleon software (Sunnyvale, CA, USA). An IEC CL31R multi-speed centrifuge employed in the plasma sample preparation was from Thermo Electron Corporation (Waltham, MA, USA).

2.5. HPLC-DAD conditions

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 (65/35 v/v) (pH 3.5, adjusted by 0.1 M HCl 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 236 nm. The injection volume was ten (10) μL.

2.6. On-line SPE conditions

Sample pretreatment was performed through an on-line SPE methodology, including a SPE cartridge MF Ph - 1 (10 mm × 4 mm, 5 μm) from Shiseido (Tokyo, Japan), 5 mM NaH_2PO_4 buffer as the loading solvent.

The system setup for on-line SPE consisted of three steps (shown in Fig. 2). In the loading step, the SPE cartridge was fitted into loading position of Valco 6-port switching valve. The plasma sample was injected directly onto the SPE cartridge by the loading pump (right pump), and the biological matrix was washed out for 2 min with the loading solvent (5 mM NaH_2PO_4 buffer, pH 3.5) at a flow rate of 1 mL/min. While the sample matrix was flushed to waste with the loading solvent delivered by the right pump, bavachinin was retained on the stationary phase of the SPE cartridge. In the transfer step, the Valco 6-port switching valve was switched to injection position that the SPE cartridge was coupled with the analytical column. Bavachinin 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 65/35 (v/v, pH 3.5) at a flow rate of 1 mL/min. In the separation step, the Valco 6-port switching valve was switched back to the loading position to equilibrate the on-line SPE cartridge with loading solvent (5 mM NaH_2PO_4 buffer, pH 3.5) at a flow rate of 1 mL/min. Bavachinin was separated in the analytical column with the chromatographic mobile phase consisted of acetonitrile–5 mM NaH_2PO_4 buffer 65/35 (v/v, pH 3.5) at a flow rate of 1 mL/min. The complete cycle of the on-line SPE loading step and chromatographic separation of the analyte was 13 min.

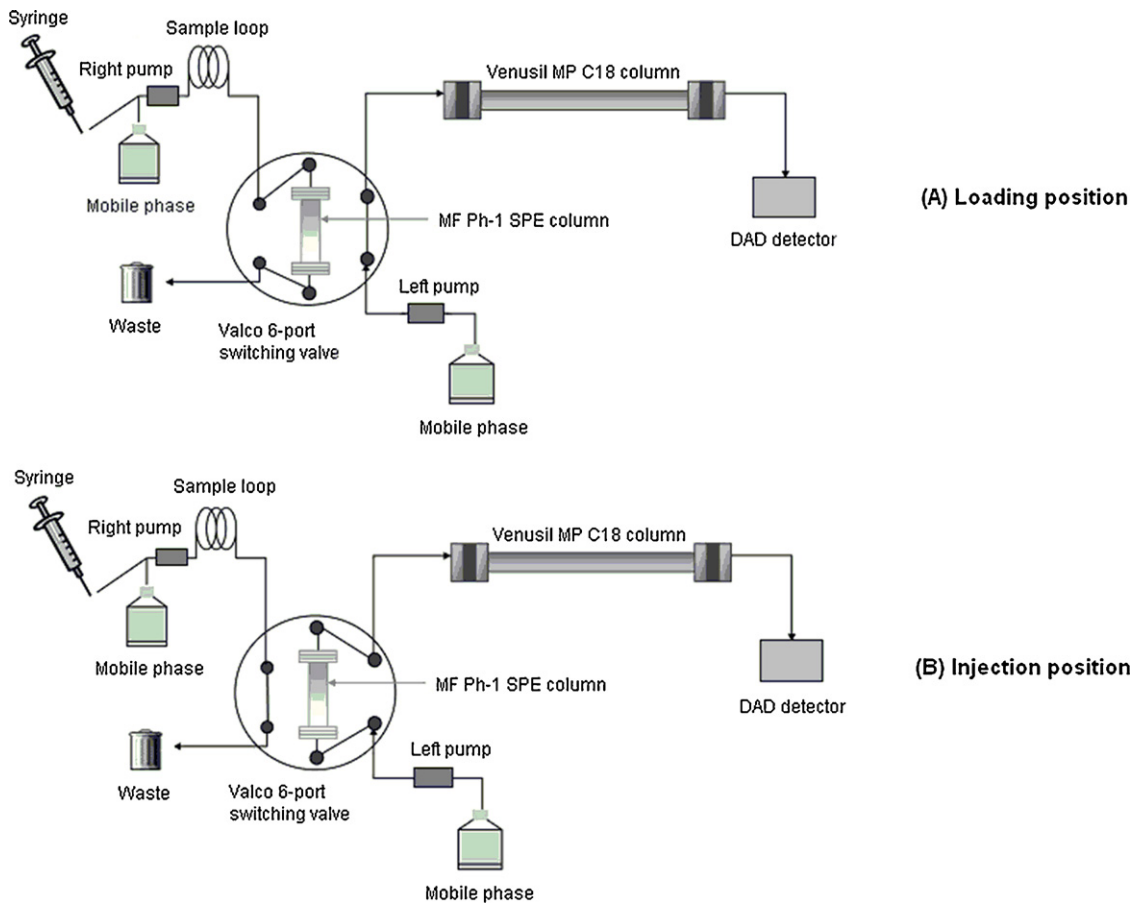

Fig. 2. The schematic diagram of on-line SPE-HPLC-DAD system.

Additionally, in order to prevent carry-over and retain full capacity, the on-line SPE cartridge was washed with acetonitrile–5 mM NaH_2PO_4 buffer (90/10 v/v) for 2 min during the separation step and then re-equilibrated with 5 mM NaH_2PO_4 buffer as initial condition (shown in Table 1).

2.7. Validation of the assay

2.7.1. Specificity and selectivity

The chromatographic interference from endogenous materials or other sources was estimated by comparing chromatograms of blank mouse plasma from six sources, plasma spiked with bavachinin, and plasma samples obtained from bavachinin PK studies.

2.7.2. Linearity and lower limit of quantification

The calibration curve was obtained by calculating the peak area of bavachinin spiked with blank plasma. Least-squares linear regression was used to estimate the linearity of the calibration standards with $1/x^2$ as the weighting factor. The lower limit of quantification (LLOQ) was determined during the evaluation of the linearity of the calibration standards. LLOQ was defined as the lowest concentration yielding a precision with coefficient of variation (CV) less than 20% and accuracy within 20% of the nominal value (i.e. accuracy between 80 and 120%) for each run analysis.

2.7.3. Precision and accuracy

The intra-day accuracy and precision were evaluated by repeated analyses of QC samples at three different concentration levels from five replicates on the same day, while the inter-day accuracy and precision were evaluated on three independent days. Accuracy was determined by calculating the percentage bias from the nominal concentration. Precision was assessed by calculating the CV for each replicates. The acceptable criteria of data induced accuracy within ±15% bias from the nominal values and a precision within ±15% relative standard deviation (RSD).

Table 1
HPLC conditions for on-line SPE-HPLC-DAD analysis.

Time (min)	Valve position	SPE cartridge			Analytical column		
		Flow rate (mL/min)	5 mM NaH_2PO_4 buffer (%)	Acetonitrile (%)	Flow rate (mL/min)	5 mM NaH_2PO_4 buffer (%)	Acetonitrile (%)
0	Loading	1	100	0	1	35	65
2	Injection	1	100	0	1	35	65
7	Injection	1	100	0	1	35	65
8	Injection	1	10	90	1	35	65
9	Injection	1	10	90	1	35	65
10	Injection	1	100	0	1	35	65
13	Loading	1	100	0	1	35	65

2.7.4. Recovery

The extraction recoveries of bavachinin were determined by comparing the peak area of the QC samples at three different concentration levels (low, middle and high) with the peak area of the corresponding standard solution in mobile phase at equivalent concentrations and expressed in percentage.

2.7.5. Stability

The stability of bavachinin in plasma was assessed by analyzing QC samples at three different concentration levels from five replicates. Freeze–thaw stability was determined after three freeze–thaw cycles; short-term temperature stability was evaluated using QC samples kept at room temperature for 24 h; long-term stability was measured using QC samples kept at −20 °C for 1 month; and autosampler stability was determined by reanalyzing QC samples in the autosampler at room temperature for 8 h after sample preparation.

2.8. Application on PK studies

Healthy BALB/c female mice (body weight: 20–22 g) were obtained from Laboratory Animal Center, Academy of Military Medical Science (Beijing, China). The animals were maintained in propylene cages (target conditions: temperature 20–25 °C, relative humidity 40–70% and 12 h dark–light cycle) with free access to standard laboratory food and water for 5 days acclimation, and then fasted the night before through 4 h after dosing for oral dosing group. The mice were divided into four dose groups randomly, they were group 1, 2, 3 and 4, 84 mice in each group, 6 mice were bled per time point. The mice in group 1, 2 and 3 were dosed with bavachinin by gavage at 25 mg/kg, 50 mg/kg and 100 mg/kg, respectively, while in group 4 were dosed via caudal vein at 25 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 (IV only), 15 min, 20 min (IV only), 30 min, 45 min, 1, 1.25 (PO only), 1.5, 2, 3 (IV only), 4, 5 (IV only), 6 (PO only), 8 (PO only), 12 (PO only) and 24 (PO only) hours post-dose. All blood samples were immediately centrifuged and obtained plasma stored frozen in a freezer set to maintain −20 °C until bio-analysis. 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 for Animal Experimentation of Nankai University (Tianjin, China).

The plasma concentrations of bavachinin were determined by using on-line SPE-HPLC-DAD method. PK parameters were determined by non compartmental methods using WinNonlin trial version 5.2 from Pharsight Corporation (Sunnyvale, CA, USA), including highest observed plasma concentration (C_{max}), time when highest plasma concentration observed (T_{max}), 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, in the absolute bioavailability study, clearance (CL) and volume of distribution at steady state (V_{ss}) were calculated after IV dosing, together with an estimate of absolute bioavailability (F). For the dose-proportionality study, an analysis of variance (ANOVA) was performed on natural log-transformed dose-normalized AUC, C_{max} and T_{max} values for bavachinin. 90% confidence intervals (CIs) for the differences between dose groups (25 mg vs 50 and 100 mg; 50 mg vs 100 mg) and standard errors associated with these differences were calculated. For normalized AUC and C_{max}, the mean difference and 90% CI were back transformed to obtain the mean ratio of treatments and 90% CI for the ratio. Data was considered to be dose proportional if the normalized AUC and C_{max} CI for the ratios were within the interval of 80–125%, and if there were no significant differences between mean T_{max}.

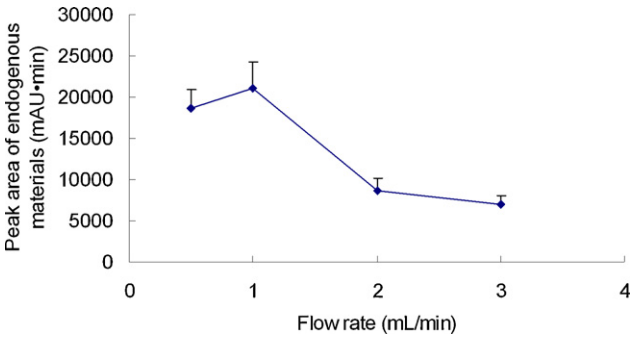

Fig. 3. Effect of flow rate of loading solvent on the peak area of endogenous materials.

3. Results and discussion

Dionex UltiMate 3000 HPLC system (Sunnyvale, CA, USA) equipped with UltiMate 3000 pump, UltiMate 3000 autosampler column compartment and UltiMate 3000 photodiode array detector was utilized for the development of the fully automated on-line SPE and HPLC system for the PK evaluation of bavachinin. The configuration of the system was shown in Fig. 2.

3.1. SPE procedure

The SPE retention and elution steps were optimized in order to carry out the pretreatment of the plasma samples containing bavachinin. 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.

3.1.1. Correlation of the flow rate of loading solvent and amount of endogenous materials

The correlation of the flow rate of loading solvent and amount of endogenous materials was investigated to search for the optimized condition to minimize the co-eluting interferences. Such tests were performed by directly connecting the SPE cartridge to the UV detector set at 210 nm, which was used to detect endogenous materials [23]. Blank plasma (10 μL) was directly injected onto the SPE cartridge with the loading solvent of 5 mM NaH_2PO_4 buffer and flow rate in a range of 0.5–3 mL/min. As shown in Fig. 3, the optimal elution of endogenous materials was achieved within 1 min when the flow rate was set at 1 mL/min.

3.1.2. Optimization of transfer time of bavachinin from the SPE cartridge to the analytical column

In transfer step, the transfer time of bavachinin from SPE cartridge to analytical column was crucial in column switching. Such tests were performed by directly injecting ten (10) μL QC samples (2000 ng/mL) from five replicates onto the SPE cartridge. After washing out for 2 min with the loading solvent (5 mM NaH_2PO_4 buffer, pH 3.5) at a flow rate of 1 mL/min, the Valco 6-port switching valve was switched to injection position that the SPE cartridge was coupled with the analytical column. Bavachinin was eluted from the SPE cartridge in the back-flush mode and transferred to the analytical column with a transfer time range of 0.5–3 min, and the chromatographic mobile phase consisted of acetonitrile–5 mM NaH_2PO_4 buffer 65/35 (v/v, pH 3.5) at a flow rate of 1 mL/min. It was observed that if the transfer time was 0.5 min, poor recovery of the analyte was obtained, if it was more than 2 min, some residual endogenous materials could be found in the chromatogram, and

the life time of the analytical column would be affected simultaneously. A transfer time of 1 min in the back-flush mode was found to be ideal for the assay.

3.2. Analytical condition

In order to achieve high sample throughput and improvement of the overall analytical quality, the analytical condition was systematically optimized.

3.2.1. Optimization of detection wavelength

The DAD detector was used in the bio-analysis to optimize chromatography for bavachinin, and the maximum absorption was found at 236 nm (shown in Fig. 1b).

3.2.2. Optimization of analytical mobile phase

In order to obtain the best separation conditions to determine bavachinin in plasma, a selective washing of the SPE cartridge was required before eluting the analytes. Thus, different concentrations of acetonitrile–5 mM NaH_2PO_4 buffer (0:100, 10:90, 20:80, 30:70, 40:60, 50:50, 65:35, 75:25, 90:10 v/v) were used as the mobile phase. The analyte signal increased with the increase of acentonitrile from 20% to 65% and reached a plateau from 65% onwards. Therefore, acetonitrile–5 mM NaH_2PO_4 buffer (65:35 v/v) was selected as the analytical mobile phase to achieve not only maximum recovery of bavachinin onto the analytical column, but also ensuring the long-term performance of the column (shown in Fig. 4a).

Similarly, different volumes (0.1–2 mL) of 0.1 M HCl solution per 1000 mL 5 mM NaH_2PO_4 buffer (pH 3.5) were selected to study its relationship with the analyte signal. The mobile phase with 1 mL of the 0.1 M HCl solution per 1000 mL 5 mM NaH_2PO_4 buffer (pH 3.5) was chosen for its high sensitivity (shown in Fig. 4b).

The influence of the mobile phase flow rate was investigated with the range of 0.5–1.5 mL/min. The best result was obtained at the flow rate of 1.0 mL/min and the separation process can be completed in a single run of 13 min with no presence of co-eluting interferences.

3.3. Specificity and selectivity

Under the condition of the present analysis, the chromatograms of the blank plasma, bavachinin reference standard spiked with blank plasma and the plasma samples of IV administrated mice were represented in Fig. 5. The retention time was 9.75 min for bavachinin, no interference from endogenous materials or other sources was found at the same retention time as the target analyte, which indicated that the elaborated procedure was specified and selective.

3.4. Linearity and lower limit of quantification

The method was linear over the concentration range of 20–4000 ng/mL, the mean values of linear regression equation was: $y = 0.0004x + 0.006$, $r = 0.9997$ ($n = 5$), where y was the peak area, x was the relative concentration and r was the correlation coefficient. The LLOQ of the assay was 20 ng/mL.

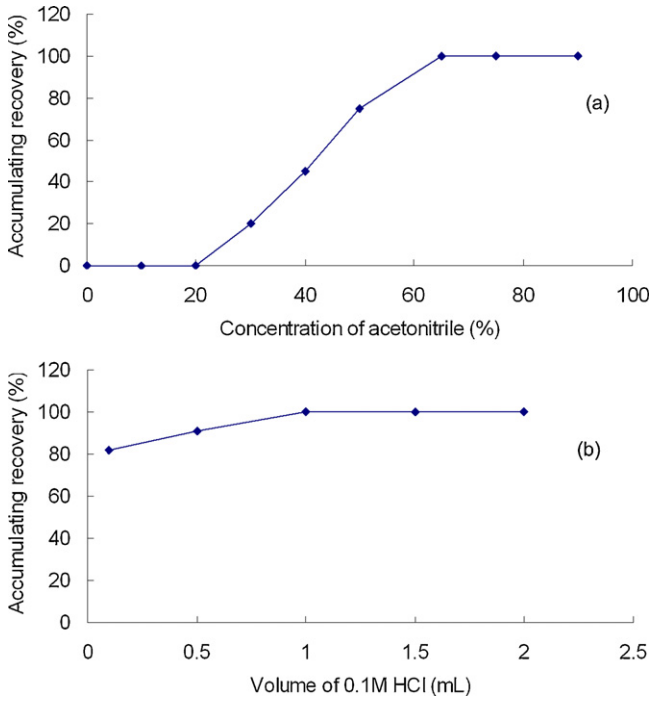

Fig. 4. Effect of (a) the acetonitrile concentration on the accumulating recovery of bavachinin and (b) the volume of 0.1 M HCl solution on the accumulating recovery of bavachinin.

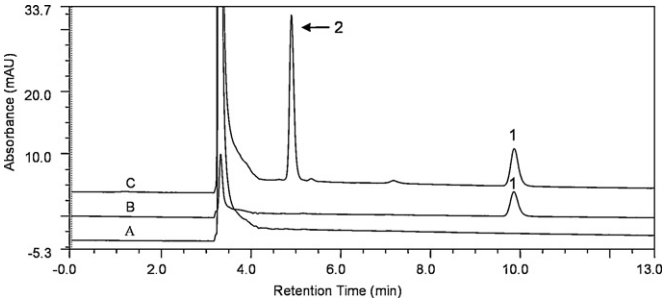

Fig. 5. HPLC chromatograms of (A) blank plasma, (B) blank plasma spiked with bavachinin, (C) plasma sample of IV administrated mice at time point of 20 min (1 bavachinin, 2 unknown).

3.5. Precision and accuracy

The intra-day and inter-day precisions and accuracies of the QC samples (40, 400 and 2000 ng/mL) were presented in Table 2. The RSD of bavachinin was in the range of 0.20–2.32% and the accuracies were between 98.47% and 102.95%, allowing the accurate assay of bavachinin in mouse plasma.

Table 2
Intro-day and inter-day precisions and accuracies of the assays ($n = 5$).

Theoretical concentration (ng/mL)	Intro-day				Inter-day			
	Concentration found (ng/Ml)		RSD (%)	Accuracy (%)	Concentration found (ng/mL)		RSD (%)	Accuracy (%)
	Mean	SD			Mean	SD		
40.00	39.92	0.87	2.17	99.81	39.39	0.91	2.32	98.47
400.00	404.39	2.28	0.56	101.10	401.72	4.37	1.09	100.43
2000.00	2059.00	4.04	0.20	102.95	2049.67	29.44	1.44	102.48

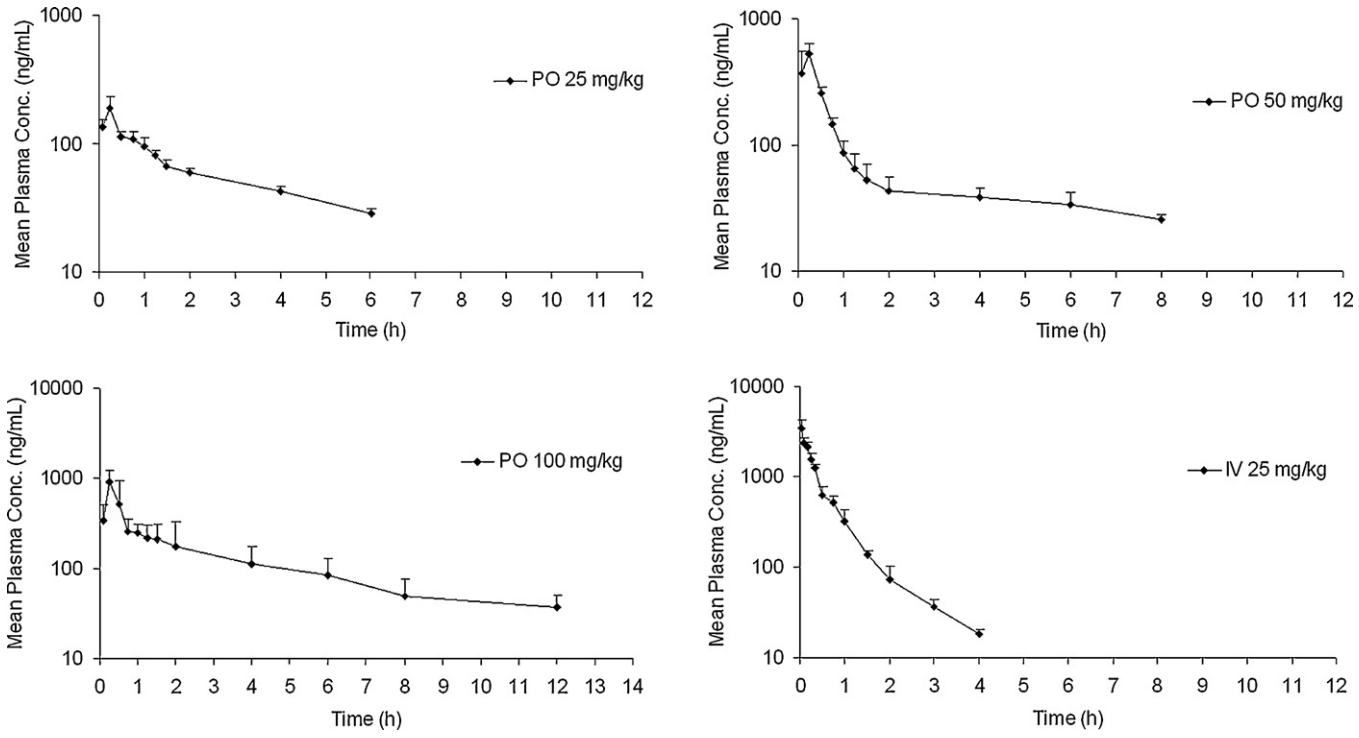

Fig. 6. Plasma concentration–time profiles of bavachinin following single oral and intravenous administration to naïve female BALB/c mice ($n = 6$).

Table 3
The extraction recovery of bavachinin from mouse plasma ($n = 5$).

Concentration spiked (ng/mL)	Extraction recovery (%)		RSD (%)
	Mean	SD	
40.00	95.80	2.20	2.29
400.00	96.37	1.47	1.53
2000.00	95.15	1.85	1.95

3.6. Recovery

The extraction recovery of the QC samples (40, 400 and 2000 ng/mL) was presented in Table 3. The recovery ranged from 95.15% to 96.37%, which indicated that the method was consistent, precise and reproducible.

3.7. Stability

No significant difference of concentrations for bavachinin in mouse plasma was observed during the 24 h period of assay, the RSD between the initial concentrations and the concentrations of bavachinin stored at −20 °C for 1 month was less than 4.11% (shown in Table 4).

The absolute concentrations of bavachinin spiked to mouse plasma were not significantly affected by the freezing and thawing test. After completion of three freezing and thawing cycles, the recoveries of bavachinin were between 95.44% and 98.14%.

3.8. Sample analysis and PK studies

In the present study, the plasma concentrations of bavachinin were successfully determined by the established method. The test article could be monitored in plasma for up to 12 h post-dose when following single oral administration at 100 mg/kg to naïve female BALB/c mice. The mean plasma concentration–time profiles were shown in Fig. 6, and the PK parameters of bavachinin were listed in Table 5. Following IV administration of bavachinin at 25 mg/kg, clearance was high (mean CL = 299.72 mL/min/kg) and was approximately 3.33-fold of hepatic blood flow. The mean volume of distribution of bavachinin was 11881.67 mL/kg, it was 16.39 times of total body water volume (725 mL/kg) [24], indicating high extravascular distribution. The mean terminal half-life following IV dosing was 0.70 h, this was reflected a tight correlation between the clearance and terminal half-life. Thus, the high systemic clearance and the associated short terminal half-life in mouse contribute to low steady-state concentrations of bavachinin observed in the present study. Following single oral administration of bavachinin solution at 25 mg/kg, 50 mg/kg and 100 mg/kg, respectively, maximum mean plasma concentrations were exhibited as 189.08 ng/mL, 527.99 ng/mL and 908.80 ng/mL, and all observed at approximately

Table 4
Stability results of bavachinin in spiked plasma samples ($n = 5$).

Theoretical concentration (ng/mL)	Short-term stability (24 h at room temperature)			Long-term stability (1 month at −20 °C)			Autosampler stability (8 h at room temperature)			Freeze–thaw stability (3 cycles)		
	Concentration found (ng/mL)		RSD (%)	Concentration found (ng/mL)		RSD (%)	Concentration found (ng/mL)		RSD (%)	Concentration found (ng/mL)		RSD (%)
	Mean	SD		Mean	SD		Mean	SD		Mean	SD	
40.00	40.43	1.35	3.35	37.30	1.53	4.11	39.60	1.44	3.65	39.26	1.42	3.63
400.00	398.93	9.38	2.35	369.52	8.40	2.27	389.43	10.50	2.70	381.78	7.69	2.01
2000.00	2033.54	47.97	2.36	1867.42	55.47	2.97	1933.52	57.64	2.98	1922.04	52.35	2.72

Table 5
Absolute bioavailability and PK parameters of bavachinin following single oral and intravenous administration to naïve female BALB/c mice ($n = 6$).

PK parameters	Intravenous (25 mg/kg)		Oral (25 mg/kg)		Oral (50 mg/kg)		Oral (100 mg/kg)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
C_{max} (ng/mL)	3408.79	817.64	189.08	40.95	527.99	112.52	908.80	332.99
T_{max} (h)	0.033	0.00	0.25	0.00	0.25	0.00	0.25	0.00
AUC_{0-t} (ng/mL h)	1408.77	285.88	365.40	40.61	538.42	118.67	1517.45	751.30
AUC_{0-INF} (ng/mL h)	1438.65	288.18	523.56	54.74	797.62	105.81	1691.60	772.78
$T_{1/2}$ (h)	0.70	0.26	3.82	0.13	7.15	2.64	4.13	1.31
MRT (h)	0.68	0.09	5.02	0.19	7.80	3.18	5.86	2.09
CL_{obs} (mL/min/kg)	299.72	61.30	–	–	–	–	–	–
$V_{ss.obs}$ (mL/kg)	11881.67	1189.43	–	–	–	–	–	–
F (%)	–	–	36.39	–	27.72	–	26.93	–

Table 6
PK parameters of bavachinin following administration of single oral doses of bavachinin ranging from 25 to 100 mg/kg. Geometric means are presented for AUC_{0-INF}, AUC_{0-t} and C_{max} ($n = 6$).

PK parameters	25 mg/kg	50 mg/kg	100 mg/kg	25 mg/kg/50 mg/kg (90% CI)	P-value	25 mg/kg/100 mg/kg (90% CI)	P-value	50 mg/kg/100 mg/kg (90% CI)	P-value
C_{max} (ng/mL)	185.49	518.44	845.44	35.78% (34.49–37.12)	0.0001	21.94% (17.33–27.78)	0.0019	61.32% (48.83–77.01)	0.0104
AUC_{0-t} (ng/mL h)	363.51	527.93	1340.27	68.86% (63.32–74.99)	0.0030	27.12% (18.63–39.48)	0.0107	39.39% (29.08–53.34)	0.0131
AUC_{0-INF} (ng/mL h)	521.10	792.08	1526.36	65.78% (60.28–71.80)	0.0005	34.14% (24.47–47.64)	0.0106	51.89% (35.95–74.91)	0.0258

0.25 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 bavachinin was 36.39%, 27.72% and 26.93%, which showed low values as well as plasma concentration, although the PO dose was high enough. The results properly indicated that a high clearance would be exhibited by bavachinin in vivo (PO dosed mouse chromatogram is shown in Fig. 7).

PK parameters of bavachinin following administration of single oral dose of bavachinin ranging from 25 to 100 mg/kg were compared in Table 6. Geometric means were presented for AUC_{0-INF}, AUC_{0-t} and C_{max}. After all doses, plasma concentrations declined after peaking in a bi-exponential manner with mean $T_{1/2}$ values of between 3.82 and 4.13 h (shown in Table 5). As shown in Table 6, when consecutive dose increments of bavachinin from 25 mg/kg to 100 mg/kg were compared, increase in C_{max} and AUC was not proportional to dose. In addition, no significant difference was observed between doses in T_{max} for the value of T_{max} for all three dose groups (25 mg, 50 mg and 100 mg) was 0.25 h.

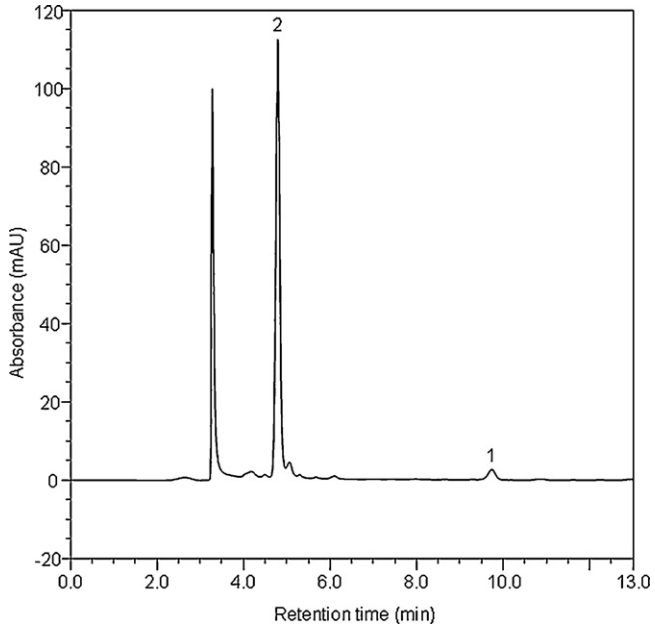

Fig. 7. Chromatogram of PO dosed (100 mg/kg) mouse at time point of 30 min (1 bavachinin, 2 unknown).

4. Conclusion

A fully automated on-line SPE-HPLC-DAD method was developed for determination of bavachinin in mouse plasma. Sample pretreatment procedures for plasma was optimized and carried out by easy-to-use and high throughput methods. Subsequently, no presence of co-eluting interferences chromatogram was obtained with a single run in 13 min, and the PK properties of bavachinin were characterized as rapid oral absorption, high clearance, and poor absolute bioavailability following single oral and intravenous administration to naïve female BALB/c mice. In conclusion, the established automated on-line SPE-HPLC-DAD method demonstrated good performance in terms of linearity, specificity, detection and quantification limits, precision and accuracy, and was successfully utilized to quantify bavachinin in mouse plasma to support the PK studies.

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