Tissue distribution study of salvianolic acid B long-circulating liposomes in mice by UPLC-MS/MS determination

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Abstract: In targeting delivery system research on salvianolic acid B, it's vital but hard to evaluate the tissue distribution for its low concentrations in tissues. So the simple, rapid, selective and sensitive UPLC-MS/MS method was provided hereby to determine the concentration of salvianolic acid B in mice tissues after intravenous administration of salvianolic acid B injections, conventional liposomes and long-circulating liposomes. The UPLC was conducted by a C_{18} column with a gradient mobile phase consisting of acetonitrile and water containing 0.1% formic acid. The tandem mass spectrometry was operated in negative-electrospray ionization selected-reaction-monitoring mode, and the optimized characteristic precursor to product ion transition m/z 717.3→519.1 was selected. The biosamples were homogenized and treated with a protein precipitation, which led to an acceptable matrix effect and extraction recovery. The linear calibration curves were plotted in the given concentration ranges. The intra-day and inter-day precisions were less than 13.9% and the accuracies were in the range of 86.3-109.2%. The tissue distribution results determined by UPLC-MS/MS we developed showed that the conventional and long-circulating liposomes we made had succeeded in prolonging the retention time and increasing the level of salvianolic acid B in certain distribution tissues such as liver, kidney and brain.

Keywords: Salvianolic acid B; UPLC-MS/MS; tissue distribution; liposome delivery system.

INTRODUCTION

Salvianolic acid B (SAB) is one of the water-soluble phenolic acids of herbal medicine *Radix Salviae Miltiorrhizae* which has been reported as an active component to reduce atherosclerosis (Chen *et al*., 2006; Joe *et al*., 2012; Lin *et al*., 2007), prevent platelet aggregation (Li *et al*., 2004), inhibit hepatic fibrosis (Gao *et al*., 2012; Li *et al*., 2012; Wang *et al*., 2012) and protect nervous system (Lee *et al*., 2013; Wang *et al*., 2010). As a potential efficient agent for clinical treatment, however, the poor chemical stability (Zhou *et al*., 2011) and bioavailability (Ma *et al*., 2007) limit the further application of SAB.

Some advanced SAB delivery systems referred to absorption enhancers (Wan *et al*., 2012; Zhou *et al*., 2009); liposomes (Isacchi *et al*., 2011; Zhang *et al*., 2012) and nanopaticles (He *et al*., 2010; Peng *et al*., 2010) are developed in recent years. It is anticipated that these new drug delivery systems could improve the stability, regulate pharmacokinetics behavior and increase the concentrations in certain tissues such as heart and brain for more efficient clinical treatment. So a laboratorial tissue distribution study in rats or mice is an important evaluation indicator for a novel SAB delivery system whether the delivery system shows a better pharmacokinetic behavior or not, thus the selective and sensitive methodology of SAB determination in tissue homogenate is essential.

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Many analytical methods have been reported to investigate the pharmacokinetic profiles of SAB in the rats (Lai *et al*., 2011; Wang *et al*., 2007; Yang *et al*., 2008), rabbits (Gao *et al*., 2009; Ma and Wang, 2007) and beagle dogs (Gao *et al*., 2009). For tissue distributions, a liquid chromatographic with ultraviolet detection recently has been described to determine SAB in different tissues of rats after oral administration (Xu *et al*., 2007), which is believed the limit of quantization is not suitable enough for the studies. Besides, more HPLC-MS/MS has been described in recent studies on the determination of SAB in biological samples for its lower limit of quantization (LOQ) of nearby 10ng/mL (Chang *et al*., 2010; Han *et al*., 2009; Zhao *et al*., 2011). However, a high-throughput and sensitive UPLC-MS/MS determination used for the tissue distribution of SAB delivery system is limited.

Therefore, the objective of this study is to assess the tissue distribution of conventional and long-circulating liposomes containing SAB by a sensitive selective UPLC-MS/MS determination, which is successful to verify the SAB in different tissues of mice.

MATERIALS AND METHODS

Chemicals and reagents

HPLC-grade methanol, acetonitrile and formic acid were purchased from Fisher scientific, Inc. (Fairlawn, NJ, USA). Analytical grade hydrochloric acid and ascorbic acid were purchased from Northern Tianyi Chemical Reagent Co. (Tianjin, China). Pure water was obtained by Milli-Q Reagent Water system (Millipore, USA).

Salvianolic acid B (SAB, Batch No. 11562-200908, purity: 96.5%) and Chloromycetin (CH, used as I.S., Batch No. 130555-200602, purity: 99.4%) were obtained from National Institutes for Food and Drug Control (NIFDC, Beijing, China). Liposomes and long-circulating liposomes containing SAB were self-made according to the article (Ma *et al*., 2011; Zhang *et al*., 2012).

Animals

Seventy-six male KM strain mice (Shanchuanhong Experimental Animal Tech Co. Ltd; Tianjin, China; weighting 30±5 g) were well housed at room temperature and provided free access to food and drinking water during the experiment with the protocol involving the care or use of animals in this study approved by TJUTCM's Institutional Animal Care and Use Committee (IACUC).

Chromatographic conditions

A Waters UPLC/MS-MS system (consisting of Acquity Binary Solvent Manager, Acquity Sample Manager, Xevo TQ, Waters, Massachusetts, USA) was applied for determination of SAB in biosample analysis.

Chromatographic separation was performed by a C_{18} column (100mm×4.6mm, 1.8 μm, Agilent Co. Ltd.) maintained at 50°C. Mobile phase consisting of (A) acetonitrile/ (B) water (containing 0.1% formic acid) was performed with an elution program as follows: 15% A (initial),15-45% A (3min), 45-50% A (1min), 50% A (1min), 50-15% A (3min).The constant flow rate was set as 0.5mL/min. A volume of 5μL prepared samples was injected into the chromatographic system with the temperature maintained at 4°C.

Mass spectrometric conditions

An Xevo TQ mass spectrometer (Waters, Massachusetts, USA) was operated in an ESI^- negative ion multiple reaction monitoring mode (MRM). The characteristic precursors $[M\text{-}H]^-$ to product ions transitions for quantification were m/z 717.3→519.1 and m/z 321.1→152.1 for SAB and I.S., respectively. Optimized conditions were as follows: Capillary voltage: 2800V, source temperature: 110°C, desolvation temperature: 350°C, desolvation gas: 1000L/h, cone gas: 12L/min, dwell time: 200ms, respectively. The cone voltage were 30V and 28V meanwhile the collision voltage of 20eV and 17eV for SAB and CH ionization, respectively. The data were acquired and processed by Mass Lynx (Version 4.1, Waters, Massachusetts, USA).

Preparation of standards solutions and quality control (QC) Samples

SAB was accurately weighted and dissolved in methanol/water (50/50, v/v) as primary stock solution at the concentration of 1mg/mL and was prepared as a series of standard or QC solutions at the desired concentrations by serial dilution. CH stock solution was also prepared at 0.1mg/mL in methanol/acetonitrile (50/50, v/v), and diluted as an I.S. working solution of 200 ng/mL. SAB and CH solutions were kept at 4°C.

10μL of the appropriate standard working solutions was added into 90μL blank plasma or blank tissue homogenate to provide calibration standards with the concentrations ranged from 10ng/mL to 2000ng/mL. Three different concentrations of QC samples represented the low, medium and high levels were 20, 500, 1600ng/mL, respectively.

Preparation of plasma and tissue homogenate sample

For plasma sample preparation, a volume of 100μL thawed plasma, 10μL of ascorbic acid solution (1.4mg/mL in water) and 10μL of methanol/water (50/50, v/v) were spiked into a neat tube and briefly vortex-mixed. 250μL of I.S. working solution was added into each tube and then vortexed for 1 min for the purpose of protein precipitation. After centrifugation at 21,380 g for 10 min, 20μL of the upper organic layer was carefully transferred into another tube, and diluted with 50μL of pure water and 130μL of I.S. working solution, then mixed. 5μL of the mixture was injected into the UPLC-MS/MS system for SAB determination.

For tissue homogenate sample preparation, probably 0.3g tissue samples (or the whole tissues if the tissue weight was below 0.3g) were thawed and cut up into a 10mL tube, then homogenized after a probable amount of 0.9% sodium chloride solution (4mL/g tissue) were added to the tube. Then a volume of 100μL homogenate was treated as the plasma preparation process described above till 5μL of each prepared sample was used for the UPLC-MS/MS analysis.

Matrix effect and extraction recovery

Matrix effects were estimated by the post-extraction addition method mentioned in the previous article (Liu *et al*., 2009). In details, absolute matrix effect (ME %), a critical evaluation of the MS signal suppression and enhancement effects, was calculated by comparing the peak areas of biosample extracts added standard solutions (B) with those of the standards in the reconstitution solvent (A) and expressed as (B/A×100%). Extraction recovery was calculated by comparing peak areas of QC samples (C) with B, which expressed as (C/B×100%). Tests were performed in three replicates on each lot of plasma and tissue samples.

Method validation

The optimized method was validated in terms of specificity, linearity, intra- and inter-day precision and accuracy, recovery and biosample stability. The specificity was evaluated by comparison among blank plasma and tissue homogenates samples; blank samples spiked with SAB and real samples after intravenous administration of SAB solutions or SAB liposome solutions.

Linearity, precision and accuracy of the method was determined by one set of calibrations and three replicates of QC samples at low, medium and high concentration within three analytical batches repeating validation. The linearity of each curve was confirmed by plotting the peak area ratio (Y) of the target compound and I.S. corresponding concentrations (C). The precision was expressed by relative standard deviation (RSD) and the accuracy was defined as comparison between the mean of the QC measurements and the known values and the formula was expressed as: [(measured concentration by the regression equations)/ (QC concentration)] × 100%. The stability of SAB was investigated in order to evaluate the possible conditions that affected the analyte during sample collection, storage and analysis. Several stability experiments were undertaken including the stability of SAB in biosamples under -80°C storage conditions, at room temperature for 4 h, after three circles of freeze and thaw, and kept in 4°C for 24h.

Tissue distribution

Seventy-six males KM mice (30±5g) were randomly divided into three groups. For group 1, sixteen mice were intravenous administered of 12mg/kg SAB solutions and four individuals were euthanized at 5, 15, 30, 60min following administration. For group 2, twenty-eight mice were intravenous administered of 12mg/kg SAB liposome solutions and four individuals were euthanized at 5, 15, 30, 60, 120, 240 and 480 min following administration meanwhile thirty-two mice were intravenous administered of 12mg/kg SAB long-circulating liposome solutions and four individuals were euthanized at 5, 15, 30, 60, 120, 240, 480 and 720 min following administration. For each point, Blood was harvested into a heparinized tube and centrifuged immediately to obtain adequate plasma for quantification, and after the mice were sacrificed, tissues (including brain, heart, liver, spleen, lung and kidney) were collected. All these biosamples were kept at -80°C until required analysis.

RESULT

Method validation

In the chromatographic conditions described before, the analytical time was 8.0 min and the retention times of SAB and CH were 4.16±0.1min and 4.90±0.1min, respectively. The chromatograms of blank samples, blank samples spiked with SAB, and rat plasma and liver samples after intravenous administration of SAB were shown in fig. 1. There were no interfering peaks at the retention time of SAB and CH, which indicated an acceptable selectivity in the conditions.

The matrix effect and extraction recovery listed in table 1, displayed the good extraction recovery of the assay and no interferences from endogenous components.

The calibration curves of the peak area (Y) to the concentration (C) were constructed by the linear least-square regression with $1/C^2$ as weighting factor. The calibration curves, correlation coefficients and linear ranges of SAB in plasma and tissues presented in table 2, showed a good linearity (R^2>0.995) over concentration ranges of SAB in plasma and other tissues.

The current method we developed provided an LLOQ of 10ng/mL for the determination of SAB in plasma, liver and other tissues, respectively. The LLOQ was sufficient for investigating tissue distribution following SAB intravenous administration in a dosage of 12mg/Kg.

The precision and accuracy of the assay were estimated and shown in table 3. The intra- and inter- day precision were all less than 13.89% and the accuracy were ranged from 86.33% to 109.22%, which indicated that the SAB assay we carried out performed the correspondent accuracy, reliability and repeatability. SAB was stable in rat plasma for no more than three months when stored at -80°C and after three freeze-thaw cycles. However, SAB decreased in prepared samples after 4h in 4°C Acquity sample manager, therefore batch processing samples were limited to prevent the decrease.

Tissue distribution study

The concentrations of SAB in various tissues after intravenous administration of three formulations were shown in table 4-6. In table 4, the concentration in plasma and all the other collected tissues (hardly detected in heart and brain) appeared at 5min post-treatment and decreased rapidly in 60min. In table 5, the C_{max} appeared at 5min in plasma (25.30μg/mL), lung (0.806μg/g), kidney (0.843μg/g) and brain (0.145μg/g), while 30min in liver (1.079μg/g) and spleen (0.972μg/g). In table 6, the C_{max} appeared at 5min in plasma (42.58μg/mL), liver (0.404μg/g), spleen (0.536μg/g), lung (1.164μg/g), meanwhile at 120min in kidney (0.183μg/g) and 4h in brain (0.185μg/g).

The mean concentration-time profile of each tissue for the three formulations was presented in fig. 2. There was no concentration-time profile of heart, because most heart homogenate sample concentrations, for all the three groups, were hardly detected and quantified accurately, which showed that there was almost not enough SAB distribution in heart.

DISCUSSION

UPLC-MS/MS optimization

By injecting the standard solutions of SAB and CH (IS), the ESI positive and negative ion modes were scanned. In these two modes, a negative ion mode was selected, of which the base peak intensity was higher than that of the positive ion. SAB yielded a quasimolecular ion with an

Table 1: The matrix effect and extraction recovery for assay of SAB in different tissues of mice

Tissue	Compound	Matrix effect (%)	Extraction recovery (%)
Plasma	SAB	95.94	70.73
	CH	100.74	101.52
Heart	SAB	109.80	70.73
	CH	105.82	102.15
Liver	SAB	107.44	79.92
	CH	95.52	99.39
Spleen	SAB	107.76	79.43
	CH	110.87	106.82
Lung	SAB	109.91	72.73
	CH	106.36	103.10
Kidney	SAB	104.76	77.32
	CH	102.05	103.04
Brain	SAB	110.09	93.91
	CH	97.83	108.21

Table 2: The calibration curves, correlation coefficient (R^2) and linear ranges for assay of SAB in different tissues of mice (n=3)

Tissue	Equation	R^2	Linear Range (ng/mL)
Plasma	Y=0.00007C+0.00142	0.9975	100-50000
Heart	Y=0.00029C+0.01719	0.9996	10-2000
Liver	Y=0.00057C+0.00133	0.9997	10-2000
Spleen	Y=0.00032C+0.00271	0.9999	10-2000
Lung	Y=0.00031C+0.00314	0.9992	10-2000
Kidney	Y=0.00027C+0.00189	0.9997	10-2000
Brain	Y=0.00035X+0.00613	0.9982	10-2000

Table 3: The precision and accuracy for assay of SAB in different tissues of mice

Biosamples (ng/mL)	QC conc (ng/mL)	Intra-day (n=3)		Inter-day (n=9)	
		Precision (RSD%)	Accuracy (mean %)	Precision (RSD %)	Accuracy (mean %)
Plasma	200	10.15	96.95	10.37	93.66
	10000	3.49	100.62	4.37	97.86
	40000	8.34	104.92	2.00	94.78
Heart	20	3.61	104.50	13.77	93.83
	500	3.80	102.37	4.29	105.18
	1600	0.68	99.02	4.03	102.78
Liver	20	10.02	94.33	5.83	97.67
	500	2.22	98.85	9.30	98.34
	1600	3.57	108.58	10.17	102.31
Spleen	20	1.13	92.33	5.09	94.67
	500	5.10	99.25	3.48	98.68
	1600	0.10	106.84	4.94	100.62
Lung	20	2.36	106.83	13.89	109.22
	500	1.25	101.85	6.06	95.21
	1600	2.03	99.99	3.94	98.59
Kidney	20	6.96	86.33	8.26	95.56
	500	2.85	98.52	2.92	99.99
	1600	3.99	102.04	3.48	100.34
Brain	20	4.97	106.00	11.52	96.33
	500	4.57	98.63	4.94	104.19
	1600	5.54	97.91	3.43	99.67

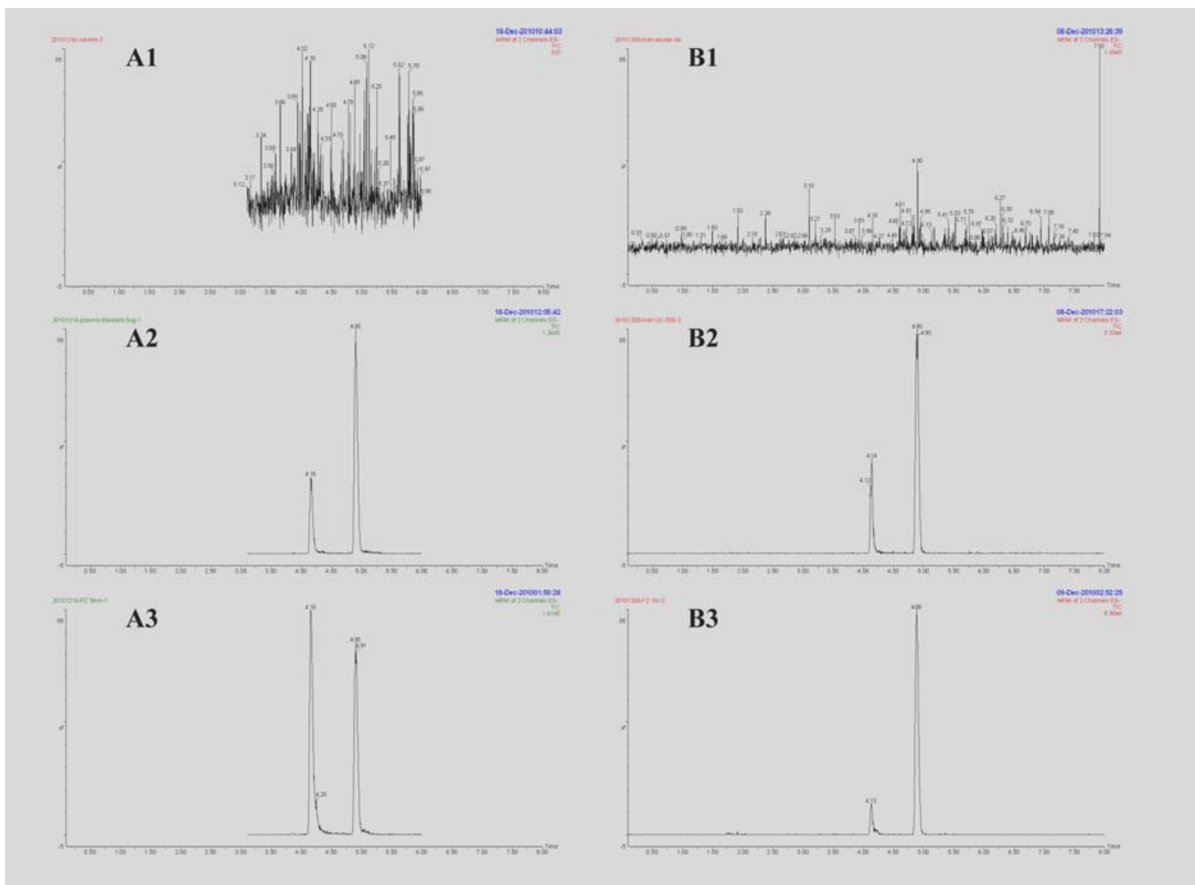

Fig. 1: Representative chromatograms of SAB (4.16 min) and CH (4.90 min) obtained by extraction of blank mice plasma (A1) and blank mice liver homogenates (B1), plasma spiked with SAB (5 µg/mL, A2) and Liver homogenates spiked with SAB (500ng/mL, B2), plasma (A3) and liver homogenates (B3) obtained 5 min and 60 min after intravenous administration of SAB liposome solution (2 mg/kg) to mice, respectively.

Table 4: SAB concentration (µg/g, for plasma the unit is µg/mL) in different tissues after i.v. administration of SAB solution to mice (Dose : 12mg/Kg, n=4)

Time (min)	Plasma (µg/mL)	Heart	Liver	Spleen	Lung	Kidney	Brain
5	1.15±0.77	nd	0.237±0.138	0.376±0.238	0.466±0.299	0.108±0.078	nd
15	1.04±0.66	nd	0.111±0.035	0.144±0.074	0.137±0.090	nd	nd
30	0.41±0.14	nd	0.155±0.104	0.734±0.287	0.064±0.027	nd	nd
60	0.34±0.27	nd	nd	0.073±0.047	nd	nd	nd

Table 5: SAB concentration (µg/g, for plasma the unit is µg/mL) in different tissues after i.v. administration of SAB liposomes to mice (Dose: 12mg/Kg, n=4)

Time (min)	Plasma (µg/mL)	Heart	Liver	Spleen	Lung	Kidney	Brain
5	25.30±2.62	nd	0.842±0.435	0.605±0.576	0.806±0.155	0.843±0.273	0.145±0.132
15	15.09±9.63	nd	0.928±0.201	0.727±0.344	0.345±0.125	0.795±0.420	0.038±0.022
30	13.36±3.85	nd	1.079±0.188	0.902±0.448	0.421±0.105	0.431±0.107	0.045±0.025
60	5.11±2.81	nd	0.549±0.335	0.291±0.257	0.096±0.039	0.160±0.116	0.123±0.175
120	4.86±1.09	nd	0.324±0.040	0.532±0.474	0.091±0.009	0.161±0.114	0.033±0.022
240	2.08±1.47	nd	0.106±0.102	0.214±0.131	0.128±0.081	0.075±0.055	nd
480	0.16±0.13	nd	nd	0.177±0.209	0.033±0.015	nd	nd

m/z 717.3 [M-H]⁻, while the IS molecule showed a quasimolecular ion with m/z 321.1 [M-H]⁻ and these ions were chosen as parent ions for fragmentation in the MRM mode. The product ions m/z 519.1 and m/z 152.1 were selected as the target ions of SAB and IS, respectively. The collision energy in the UPLC-MS/MS mode was investigated to optimize the sensitivity and the optimal values were found to be 20 and 17 eV for SAB and IS, respectively.

The LC mobile phase was optimized with varying percentages of organic solvent and different modifiers in water. Acetonitrile was used as an alternative, although peak shape was improved the ionization efficiency was suppressed and sensitivity compromised.

Sample preparation

Liquid-liquid extraction and protein precipitation are the commonly used methods of sample preparation. In our work, protein precipitation was chosen because the convenient process, resulting in good extraction recovery and no interferences from endogenous components (table. 1), could simplify the whole sample preparation procedure which is essential for high throughput and avoid the SAB oxidization during the procedure. The ascorbic acid was also added in the homogenate to prevent the oxidization of SAB.

Tissue distribution study

In mice intravenously administered by SAB solution, SAB appeared and decreased rapidly. Liver, spleen and lung were the main target tissues after SAB solution injected.

Compared with SAB solution, the increased C_{max} in mice treated with SAB liposomes indicated that the liposome injection obviously increased the distribution in the main tissues especially in liver and spleen because of the uptake of liposomes by macrophages such as Kupffer cells (Ishida *et al*., 2009). Besides, the liposomal formulation had obviously prolonged the SAB retention time in main tissues from 60min to 240-480min.

In mice intravenously administered by SAB long-circulating (PEGylated) liposome, SAB was prolonged by the formulation of PEGylated liposomes, and sustained a certain concentration over 720min. Compared with the liposome solutions in different tissues, SAB in PEGlytated liposomes was more distributed in plasma and lung, but less distributed in liver, spleen and kidney, which showed that the PEGylated liposome had significantly protected the SAB from metabolism, biotransformation and elimination of body (Isacchi *et al*., 2011). In our experiment, SAB in brain could not be detected in the solution group but reach a certain concentration in two liposome groups, suggesting that the drug delivery of liposomes efficiently enabled SAB to permeate across the blood-brain barrier (Gaillard *et al*., 2012).

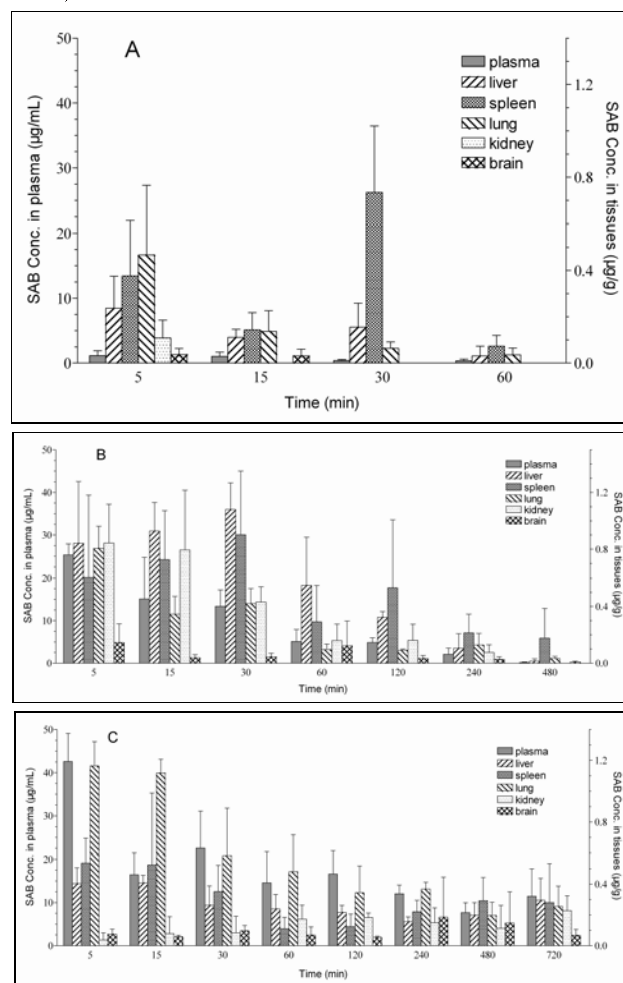

Fig. 2: The mean concentration-time profiles of SAB in plasma different tissues following intravenous administration of a single 12mg/Kg dose of (A) SAB injection, (B) SAB liposomes and (C) SAB long-circulating liposomes to mice (Each point represents the mean ± SD of 4 mice)

CONCLUSIONS

A simple, rapid and reliable UPLC-MS/MS method was developed in the article for the determination of SAB in plasma and different tissues of mice, and was successfully utilized in the tissue distribution studies of conventional and long-circulating liposomes containing SAB after intravenous administration. In our opinion, it is the first report to assay SAB in tissue samples by UPLC-MS/MS determination. The results of tissue distributions also showed that the conventional and long-circulating liposomes we made containing SAB had succeeded in prolonging the retention time and increasing the level of SAB in major distribution tissues such as liver, kidney and brain.

Table 6: SAB concentration (μg/g, for plasma the unit is μg/mL) in different tissues after i.v. administration of SAB long-circulating liposomes to mice (Dose: 12mg/Kg, n=4)

Time (min)	Plasma (μg/mL)	Heart	Liver	Spleen	Lung	Kidney	Brain
5	42.58±6.50	nd	0.404±0.101	0.536±0.159	1.164±0.158	0.037±0.047	0.075±0.032
15	16.43±4.99	nd	0.408±0.048	0.524±0.464	1.118±0.089	0.078±0.110	0.057±0.011
30	22.51±8.57	nd	0.263±0.125	0.351±0.170	0.584±0.306	0.084±0.107	0.097±0.034
60	14.59±7.12	nd	0.239±0.093	0.110±0.074	0.480±0.237	0.173±0.091	0.069±0.053
120	16.60±5.31	nd	0.216±0.046	0.123±0.083	0.344±0.173	0.183±0.029	0.054±0.008
240	12.05±2.00	nd	0.156±0.031	0.220±0.074	0.368±0.044	0.149±0.097	0.185±0.260
480	7.68±2.31	nd	0.198±0.082	0.292±0.151	0.196±0.086	0.113±0.148	0.147±0.203
720	11.47±6.35	nd	0.295±0.143	0.280±0.253	0.255±0.131	0.227±0.097	0.067±0.039

ACKNOWLEDGEMENT

This study was financially supported by National Natural Science Foundation of China (81001645), National Key Technology Research and Development Program of China (2012ZX09304007), Program for New Century Excellent Talents in University (NCET-12-1068), and supported by Program for Changjiang Scholars and Innovative Research Team in University.

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