Simultaneous quantitative analysis of multi-compounds by a single marker in *Radix Astragali* by using serum HPLC-MS feature

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Abstract: Using the serum pharmacochemistry method for Chinese Medicine, the material basis of *Radix Astragali* (RA) for "regulating and enriching blood" was studied. By compared with blank blood sample as a positive control in adult Wistar rats, four original saponinsas the material basis for "regulating and enriching blood" were absorbed into the blood after oral administration of RA. They were identified as astragaloside, astragaloside, astragaloside and astragaloside by HPLC-MS. According to the constituents absorbed into blood, the extracts of RA were prepared. In addition, the present patterns of quality control are limited to industrial application because most of the natural standard ingredients are very expensive and unavailable. Therefore, a quantitative analysis method of multi-components with a single marker (QAMS) was established and used to simultaneously measure four saponins from RA absorbed into the blood (Astragaloside, astragaloside, astragaloside and astragaloside). We used astragaloside I as the reference, the relative correction factors (*f*) of the other three saponins were measured by HPLC-MS. Within the linear ranges, the values of *f* of astragaloside I to astragaloside IV, astragaloside III and astragaloside II were 0.533, 0.779 and 0.934, respectively. According to the *f* values, we simultaneously determined four saponins using only one marker. The results of QAMS method were validated compared to that of external standard method, and no significant difference was observed.

Keywords: Constituents absorbed into blood after oral administration; The material basis; *Radix Astragali*; relative correction factor.

INTRODUCTION

Traditional Chinese medical science, including Chinese Herbal Medicine, has been gradually developed into a unique theoretical system by thousands of years of clinical practice (Fan *et al*., 2012; Wu *et al*., 2010). However, the material basis of majority of compounds from Chinese Herbal Medicine used as "active compounds" or so-called "medicinal compounds" have not been studied clearly (Chohan *et al*., 2010). Furthermore, it is known that Chinese Herbal Medicine produce efficacy only after they are transported into the blood circulation by oral administration (except for directly intestinal and external application drugs) (Wang *et al*., 2011; Wu *et al*., 2009; Yang *et al*., 2012; Singhuber *et al*., 2009). There are three kinds of the chemical state from the original traditional Chinese medicine *in vivo* before they were absorbed into the blood circulation: 1). Some chemicals were excreted with shit and urine instead of absorbed into the blood circulation; 2). Other chemicals as original components were directly absorbed into the blood circulation; 3).The other chemicals were converted into new components when they entered the blood circulation (fig. 1).

The study showed that effective substances kept in the serum, and a part of them were used as index compounds for quality assessment of TCMs. However, the present modes of quality control are limited to some industrial application because a part of the natural standard ingredients are very expensive and unavailable.

Some researchers have proposed the idea of "quantitative analysis of multi-compounds by a single marker (QAMS)" (Sun *et al*., 2012). The main contents included that the only one compound (which could be obtained easily) was used as the reference to determine other compounds (which couldn't be obtained easily) (Zhu *et al*., 2008). The principle was basically the same as the relative correction factors (*f*) method. In other words, if a Chinese Herbal Medicine sample contained the i compounds: $W_i/A_i = f_i (i=1,2,..,k,\ldots,m)$. Where W_i is the compound concentration, A_i is the compound peak area. One of the compounds (if it is k) is selected as the internal standard substance. The *f* is established by k and the other compounds. $f_{km} = f_m/f_k = W_m \times A_k / W_k \times A_m$.The quantitative calculation formula can be derived: $W_m = f_{km} \times W_k \times A_m / A_k$. Where A_k is the peak area of internal standard ingredient, W_k is the concentration of internal standard ingredient. A_m is the peak area of the other component, W_m is the concentration of the other component. According to the *f* values, we simultaneously determined multi-compounds by a single marker (Gao *et al*., 2009).

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Table 1: Description of the test samples of RA

Sample No.	Source	Species	Growth region
1	Wildness	*Astragalus membranaceus* (Fisch.) Bge.	Chongqing
2	Wildness	*Astragalus membranaceus* (Fisch.) Bge.	Shanxi
3	Wildness	*Astragalus membranaceus* (Fisch.) Bge.	Hebei
4	Wildness	*Astragalus membranaceus* (Fisch.) Bge.	Gansu
5	Wildness	*Astragalus membranaceus* (Fisch.) Bge.	Sichuan
6	Wildness	*Astragalus membranaceus* (Fisch.) Bge. Var. *mongholicus* (Beg.) Hsiao	Neimenggu

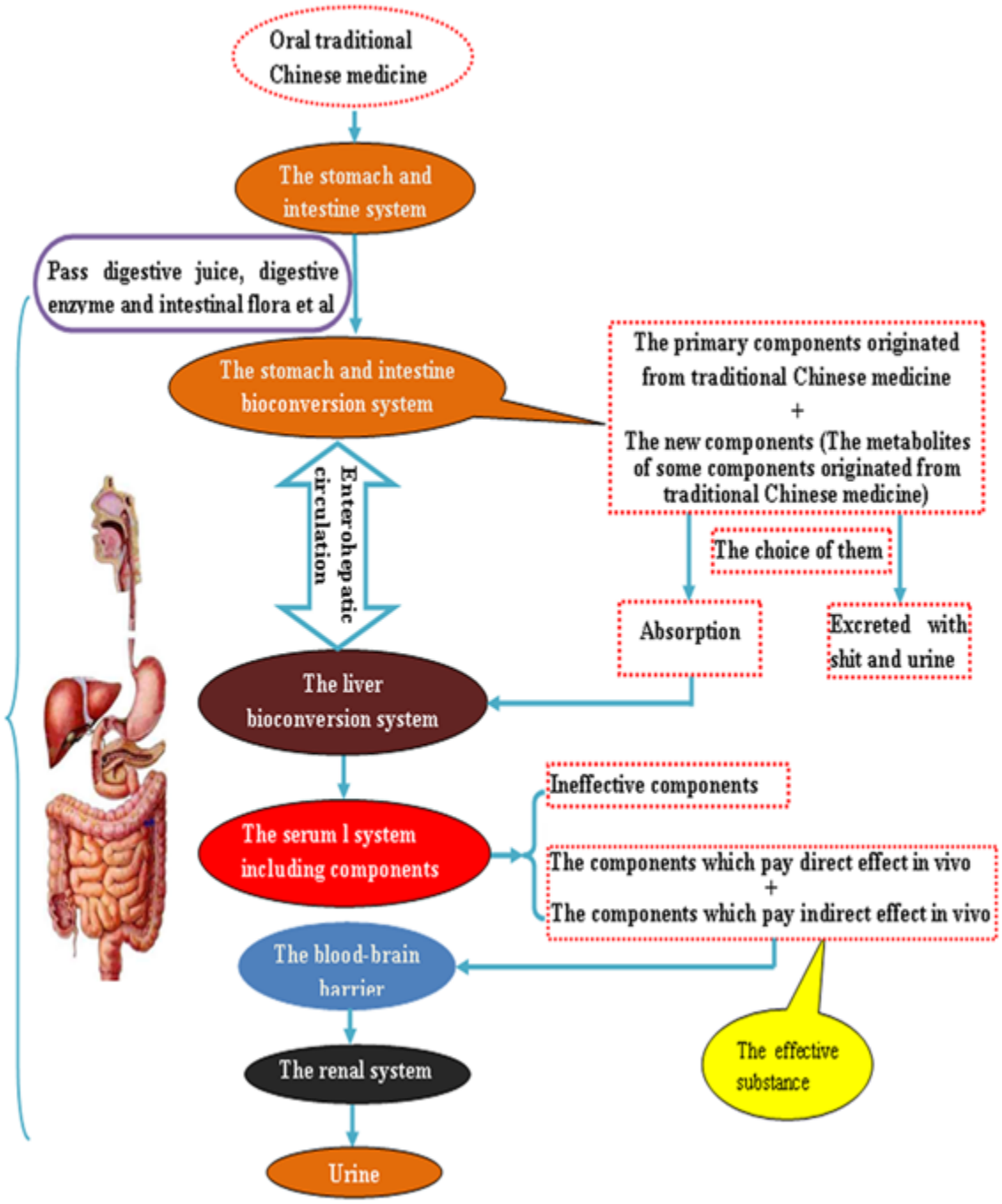

Fig. 1: The change *in vivo* about the compounds originated from oral traditional Chinese medicine.

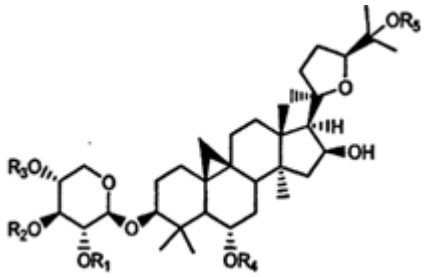

Fig. 2: Chemical structures of 4 investigated compounds in RA.

No	components	R_1	R_2	R_3	R_4	R_5
1	Astragaloside	$CH3COO^-$	$CH3COO^-$	H	$C_6H_{12}O_6$	H
2	Astragaloside	$CH3COO^-$	H	H	$C_6H_{12}O_6$	H
3	Astragaloside	$C_6H_{12}O_6$	H	H	H	H
4	Astragaloside	H	H	H	$C_6H_{12}O_6$	H

Table 2: Linearity, LOD, LOQ, and *f* value of four control compositions

Compound	Linear range (μg/ml)	Linear equation	Regression r (n=5)	LOD (μg/ml)	LOQ (μg/mL)	*f*
Astragaloside	0.031-12.40	Y=3960.3X-2136.4	r=0.9923	0.009	0.026	0.533
Astragaloside	0.089-10.68	Y=1792.1X-686.67	r=0.9949	0.013	0.037	0.779
Astragaloside	0.094-45.12	Y=854.68X+466.47	r=0.9982	0.012	0.035	0.934
Astragaloside	0.034-12.24	Y=2211.3X-1016	r=0.9933	0.011	0.034	1.0000

Table 3: Intra-day and inter-day precisions of four analytes

Analytes	Intra-day precisions (mg/g)		Inter-day precisions (mg/g)	
	Mean	R.S.D (%)	Mean	RSD (%)
Astragaloside IV	1.15	1.4	1.18	1.8
Astragaloside III	0.172	1.9	0.179	2.0
Astragaloside II	1.345	1.3	1.311	1.7
Astragaloside I	0.523	0.81	0.507	1.1

Table 4: The recovery and relativere coveries of four analytes

Analytes	Initial (μg)	Added (μg)	Detected (μg)	recovery (%)	RSD (%)
Astragaloside IV	575.14	589.31	1159.56	99.17	0.88
Astragaloside III	86.13	88.26	173.53	99.02	2.1
Astragaloside II	672.24	681.04	1351.65	99.76	1.7
Astragaloside I	261.21	270.54	534.94	100.18	0.83

Radix Astragali (Huangqi, RA) belongs to the Astragalus genus, Leguminosae family, which has been reported to be effective for "regulating and enriching blood" included improving the immune system, the respiratory systemand the circulatory system and helpful in regeneration of the tissue (National Commission of Chinese, 2010; Chan *et al*., 2009; Yang *et al*., 2009; Yan *et al*., 2010; Xiao *et al*., 2009). Some previous studies showed saponins and flavonoids are the most important active components (Yan *et al*., 2010). At present, the researches on quality evaluation of RA are mainly focused on fingerprint characteristic features. Quantitative analysis of main chemical compositions in RA had not been reported except for astragaloside IV (Huang *et al*., 2009; Chu *et al*., 2010; Qi *et al*., 2009). By using the serum pharmacochemistry method of Chinese Medicine, the material basis of RA for "regulating and enriching blood" was studied, and we reported quantitative analysis of main saponins compounds absorbed into blood after oral administration of RA.

MATERIALS AND METHODS

Materials

Six representative samples of RA were provided by Chongqing Academy of Chinese Materia Medica, Professor Chang-hua Wang (table 1) identified the herbs. Astragaloside, astragaloside, astragaloside and astragaloside were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Their structures are shown in fig. 2. Chromatography-grade acetonitrile applied for HPLC-MS

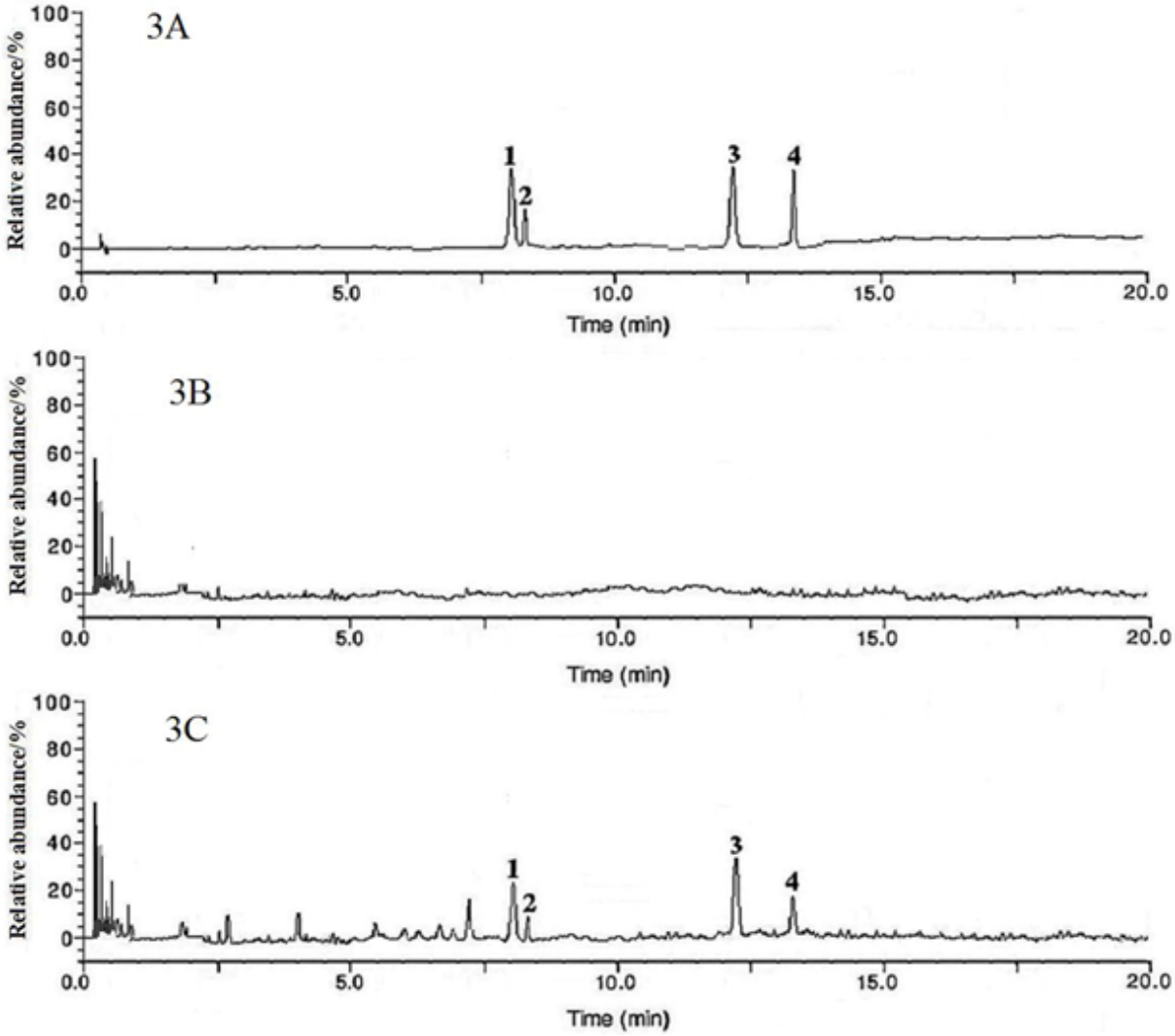

Fig. 3: HPLC-MS chromatograms of the standard solutions (3A), blank serum (3B) and extracts in RA (3C, Chongqing). Astragaloside IV (1), Astragaloside III (2), Astragaloside II (3), Astragaloside I (4), Formononetin (5).

analysis were purchased from Alltech Scientific (Beijing, China). HPLC-grade water was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Formic acid was A.R.-grade and bought from the Chongqing Chemical Company (Chongqing, China). All other chemicals applied in the studies were analytical grade reagents and bought from Promega Corporation (Madison, WI, USA).

Animals

Adult Wistar rats (180-220g) were housed in temperature-controlled rooms with access to food and water adlibitum until they were used.

Methods

Preparation of RA

RA was prepared by using traditional methods. Briefly, the dried RA powders were extracted by refluxing for 2.0h with 10 volumes of 80% ethanol. After 2.0h, the solution was filtered and the residue was re-extracted twice with the same volume of 80% ethanol. At last, the entire filtered solution of mixture was concentrated and dried with a spray dryer. The final samples were stored.

Dosage regimen design

According to Chinese Pharmacopoeia recommendations (2010 edition) for the safe and effective dosage of RA, adult Wistar rats were administered by 1.00g/kg of RA. All doses were administered by intragastrically.

Preparation of blank serum sample and RA serum sample

Twenty adult Wistar rats (half male and half female) were equally and randomly divided into a control group and a medicated group (n=10 for each group). The control group received an equivalent volume of saline (i.g) while the medicated groups received treatment as outlined in

Section Dosage regimen design. After 60min, the control and medicated group were anaesthetised by diethyl ether, the blood were taken by femoral artery intubation blood. The collected blood were centrifuged at 3000 rpm for 10 min at 4°C; then 400µl upper solution was collected.2ml methanol was added, and mixed, After 30min' standing, the upper solution was selected and dried.100µl was added to dissolve and centrifuged at 3000rpm for 10min again and prior to HPLC injection.

Preparation of standard solutions

The standard stock solutions of the five compounds were directly prepared with methanol. The standard solutions containing the four compounds were prepared and diluted with methanol to appropriate concentrations for establishment of calibration curves. The standard stock solutions were all prepared in the dark brown flasks.

Chemical analysis of active compositions by HPLC-MS

The HPLC analyses were applied by using a Waters HPLC system and a Waters Acquity TM BEH C_{18} analytical chromatographic column (150 mm × 2.1 mm × 1.7µm) in the study. The mobile phase was originally composed of acetonitrile (component A) and 0.1% formic acid solution (component B), where it was held for 2 minutes. From 3 to 8 minutes, the concentration of component A was increased linearly to 66%, where it was held for 4 minutes. From 13 to 17 minutes, the concentration of A was linearly decreased from 66% down to 32%, where it was held for 3 minutes. The mobile phase flow rate was 0.2ml/min, and the injection volume was 10µL.

The MS system was composed of a Quattro LC triple quadrupole tandem mass spectrometer with the electro spray ionisation (ESI) source. The ESI source was executed at a source voltage of 3.6 kV and with a tube compensation lens voltage of 32 V. Nitrogen was used for both the sheath gas (precolumn pressure: 7Mpa) and the auxiliary gas (precolumn pressure: 0.56Mpa, flow rate: 10L/min). The capillary was heated to 330°C and maintained within a voltage range of 16 to 52 V. Using the full-scan and positive-ion mode, we monitored ions in the 100 to 1000 m/z range. HPLC-MS chromatograms of the standards and RA sample are shown in figs. 3A and 3B, respectively.

RESULTS

Astragaloside I was chosen as the internal standard because of its good separation from the other ingredients in the chromatogram. To verify the feasibility of this newly established method, the classic external standard method for the measure of other three saponins (astragaloside, astragaloside III, astragaloside IV) was also carried out at the same time. The *f* was used to measure any differences between these two methods.

Optimisation of HPLC-MS conditions

The use of 1.7-µm porous particles packed into a short (150 mm×2.1mm) C_{18} column at a flow rate of 0.2mL/min yielded an improved resolution next to peaks. The four standard of saponins by collecting the MS spectra in full-scan mode were inspected. Anterior studies have shown that this positive ion mode produces prominent $[M+H]^+$ ions in the first order mass spectra of the four astragalosides with higher sensitivity than negative ionisation [19]. Therefore, $[M+H]^+$ ions were chosen as the quantitation ions in the study. Source voltage also indicated to be an important factor in the quality of MS spectra obtained. Out of 3.0, 3.5, and 4.0 kV used in the positive MS scan mode, the source voltage of 3.5 kV gave a higher signal to noise ratio (S/N) for each of the four compounds. Finally, setting the capillary voltage to 15 to 50 V further increased sensitivity and yielded a complete fragmentation pattern for all four astragalosides.

Preparation of RA extract and quantitative analysis of the main compounds

Calibration curve linearity was determined by using standard solutions. The linear regression equations measured by plotting peak area (Y) versus concentration (X) for each ingredient are given in table 2. All calibration curves showed good linearity ($r^2 \geq 0.99$) within the concentration ranges tested.

The limits of quantification (LOQ) and detection (LOD) were defined as the concentrations of a compound with signal-to-noise ratios (S/N) of 10:1 and 3:1, respectively. Five ingredients were determined by serial dilution of a standard solution by using the described HPLC-MS/MS conditions. The LOQs and the LODs for the ingredients were found to be less than 0.095µg/L (LOQ) and 0.031µg/L (LOD) for all five ingredients under the described HPLC-MS/MS conditions (table 2).

To verify the validity of this newly established method, validation tests for precision and accuracy were performed. Intra-day precision for each marker ingredient at one concentration level is listed in table3. The RSDs of the intra-assay were in the range of 0.81-1.9% for HPLC-MS/MS. Inter-day precision for each marker ingredient at one concentration level was also investigated with RSDs in the range of 1.1-2.0% as listed in table 3. All these data indicated that the described method had an accepted degree of precision.

To test the recoveries of the methods, six portions of RA were spiked with the mixed standard solution. The samples were handled as described above, and the results are collected in table 4. All recoveries were between 98.84% and 100.18%, with RSDs of 2.1% or less, which is well within acceptable limits (table 4).

Table 5: The quantities of four analytes from the six batches of *Radix Astragali*. samples

No	Ingredients	Content (mg/g; n=3)		
		The external standard method	*f* value method	Relative deviation (%)[a]
1	Astragaloside IV	1.168	1.171	-0.26
	Astragaloside III	0.171	0.169	1.18
	Astragaloside II	1.311	1.305	0.46
	Astragaloside I	0.524	0.522	0.38
2	Astragaloside IV	1.153	1.164	-0.95
	Astragaloside III	0.221	0.218	1.38
	Astragaloside II	1.242	1.241	0.08
	Astragaloside I	0.742	0.737	0.68
3	Astragaloside IV	0.902	0.901	0.11
	Astragaloside III	0.116	0.115	0.87
	Astragaloside II	1.211	1.225	-1.14
	Astragaloside I	0.851	0.848	0.35
4	Astragaloside IV	1.307	1.284	1.79
	Astragaloside III	0.104	0.102	1.96
	Astragaloside II	1.282	1.273	0.71
	Astragaloside I	0.768	0.764	0.52
5	Astragaloside IV	0.922	0.916	0.66
	Astragaloside III	0.0921	0.0917	0.44
	Astragaloside II	1.492	1.468	1.63
	Astragaloside I	0.458	0.464	-1.29
6	Astragaloside IV	1.031	1.026	0.49
	Astragaloside III	0.151	0.149	1.34
	Astragaloside II	1.048	1.037	1.06
	Astragaloside I	0.771	0.766	0.65

[a]Relative deviation= (content determined by the external standard method- content determined by *f* value method) / content determined by *f* value method.

DISCUSSION

Selection of obtained time in preparation of serum sample containing RA

According to Section "Preparation of blank serum sample and RA serum sample", the serum samples containing astragaloside, astragaloside, astragaloside and astragaloside absorbed into blood after oral administration of RA, were gained different time (10, 20, 30, 60 and 120min) to optimise the gained productivity. The determination results of the four investigated compounds were calculated according to Section" Chemical analysis of active compositions by HPLC-MS". The results indicated that their values increased rapidly between 10 and 30 minutes, and slowly increased again until 60 minutes. After 60 minutes, the values decreased as the increase in extraction time. The results of four investigated ingredients reached their highest values at 60 minutes, which was the length of extraction time used in subsequent experiments.

Application of optimised method

To verify the method, six batches of RA were determined by using the optimised parameters described in Section" Preparation of blank serum sample and RA serum sample". table 5 shows that all of the four saponins were reliable in the different batches. Out of the four ingredients, the average content of astragaloside was the highest, following astragaloside, and astragaloside III as the lowest one. The average content of saponins in the *RA* samples fluctuated according to the source of the raw materials, treatment of which can vary due to several factors (e.g., cultivation or storage) (Wang *et al*., 2009).

CONCLUSION

A rapid, sensitive and reliable method for the simultaneous quantitative determination of four saponins ingredients from RA using one marker with HPLC-MS based on serum fingerprint feature has been developed. Our results showed that it is a good alternative technique and offers many analytical advantages (e.g. extracting and pre-concentrating analytes of the effective substance) over traditional methods. With good reproducibility, low detection limits, and a wide range of linearity, the method can be used for the quality control of RA samples.

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