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A rapid and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of hydroxysafflor yellow A in human plasma: Application to a pharmacokinetic study

AiDong Wen[a,*], Jing Yang[a], YanYan Jia[a], ZhiFu Yang[a,*], Yun Tian[a], Yin Wu[a], ZhiRui Wang[a], ZhongGui He[b]

[a] Department of pharmacy, Xijing Hospital, Fourth Military Medical University, Shaanxi, China
[b] Department of Bio-pharmaceutics, School of Pharmacy Shenyang Pharmaceutical University, Shenyang, China

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ABSTRACT

A sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the determination of hydroxysafflor yellow A (HSYA) in human plasma. HSYA was extracted from human plasma by using solid-phase extraction technique. Puerarin was used as the internal standard. A Shim-pack VP-ODS C_{18} (150 mm × 4.6 mm, 5 μm) column and isocratic elution system composing of methanol and 5 mM ammonium acetate (80:20, v/v) provided chromatographic separation of analytes followed by detection with mass spectrometry. The mass transition ion-pair was followed as *m/z* 611.19 → 491.19 for HSYA and *m/z* 415.19 → 295.10 for puerarin. The proposed method has been validated with a linear range of 1–1000 ng/ml for HSYA with a correlation coefficient ≥0.999. The lower limit of quantitation was 1 ng/ml. The intra-batch and inter-batch precision and accuracy were within 10%. The average extraction recovery was 81.7%. The total run time was 5.5 min. The validated method was successfully applied to the study on pharmacokinetics of HSYA in 12 healthy volunteers after a single oral administration of safflower oral solution containing 140 mg of HSYA.

1. Introduction

The dried flower of the safflower plant, *Carthamus tinctorius* L. has been used extensively to treat coronary heart disease, hypertension, and cerebrovascular disease [1]. In traditional therapy with Chinese medicine, the whole safflower is orally administrated in the way of decoction. Safflower oral solution, the safflower water-soluble extract, was used for the treatment of stroke, coronary heart disease and angina pectoris which are due to blood stasis. Phytochemical investigation shows that safflor yellow (SY) is the main constituent in water-soluble extract of safflower [2]. Hydroxysafflor yellow A (HSYA) (Fig. 1(A)), the main active component of SY, has been demonstrated to have good pharmacological activities of antioxidation, myocardial and cerebral protective and neuroprotective effects [3–7]. HSYA is chosen as an active marker component for controlling the quality of safflower in Chinese Pharmacopoeia (The State Pharmacopoeia Commission of China. 2005). Therefore, HSYA was chosen as index of safflower oral solution in our study.

Some high-performance liquid chromatography methods employing UV detection have previously been developed to determine HSYA in rat plasma [8,9]. However, the lower limit of quantification (LLOQ ≥ 0.046 μg/ml) of those methods coupled with UV detection is too high to monitor the therapeutic levels of safflower oral solution. There has been no report developing the method for the determination of HSYA in human plasma and studying the pharmacokinetics of HSYA in human by now. Therefore, it is important to investigate the pharmacokinetic characteristic of HSYA in human for reasonable clinical application. In order to evaluate the pharmacokinetic character of HSYA after administration of safflower oral solution, a selective and sensitive assay method is needed for its quantification in human plasma. In this study, a rapid and sensitive LC–MS/MS method was developed and validated for the determination of HSYA in human plasma. The method exhibited excellent performance in terms of high selectivity, wide linear range (1–1000 ng/ml), short run time (5.5 min per sample), low LLOQ (1 ng/ml) and small injection volume (10 μl). The method was successfully applied to study the pharmacokinetics of HSYA in human.

* Corresponding author. Tel.: +86 29 84775475 fax: +86 29 84773636.
E-mail address: adwen-2004@hotmail.com (A. Wen).

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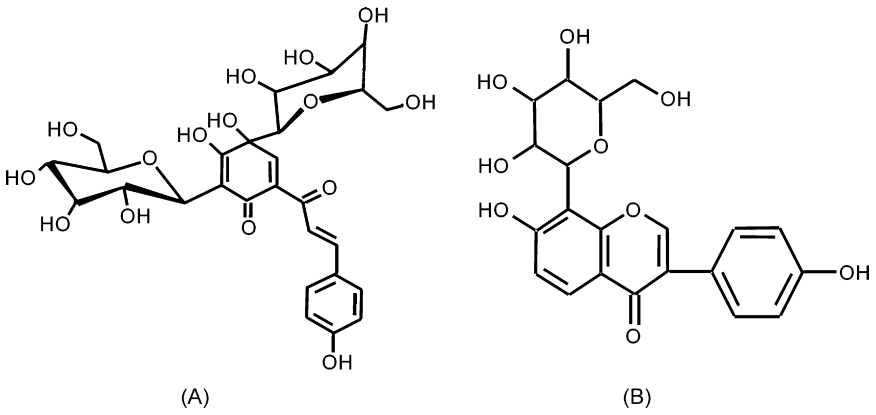

Fig. 1. The chemical structure of HSYA (A) and internal standard puerarin (B).

2. Experimental

2.1. Reagent and materials

Safflower oral solution was obtained from Yongning pharmaceutical Co., Ltd. (Zhejiang, China). HSYA reference standard (98.0% purity) was provided by Shandong Lvye Natural Medicine Research and Development Center (Shandong, China). Puerarin, the internal standard (IS, 98.2% purity), was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Methanol of HPLC-grade was purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). Other chemicals were all of analytical grade. Drug-free human plasma from healthy volunteers was kindly provided by the Blood Center of Xijing Hospital (Shaanxi, China) and was stored at −20 °C.

2.2. Instrumentation

The LC–MS/MS system was performed using an Agilent 1200 series HPLC and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA). The chromatographic separation was achieved on a Shim-pack VP-ODS C_{18} (150 mm × 4.6 mm, 5 μm) column. All data were acquired employing Agilent 6410 Quantitative Analysis version analyst data processing software.

2.3. Chromatographic conditions

The mobile phase was a mixture of methanol 5 mM ammonium acetate solution (80:20, v/v), which was pumped at a flow rate of 0.4 ml/min. The column oven temperature was set at 30 °C. The total run time was 5.5 min. The sample injection volume was 10.0 μl. Mass spectrometric detection was performed on a Series 6410 Triple Quad LC–MS/MS (Agilent Technologies, USA) using multiple reaction monitoring (MRM). A turbo electrospray interface in negative mode was used. The main working parameters of the mass spectrometer are summarized in Table 1.

2.4. Sample preparation

An aliquot of 1 ml human plasma sample was mixed with 50 μl of internal standard working solution (100 ng/ml). The mixture vortexed for 30 s and then directly loaded onto a Cleanert ODS cartridge (500 mg/3 ml) that was previously conditioned with 2 ml methanol followed by 2 ml distilled water. Plasma sample was drained out under vacuum, followed by washing cartridge with 1 ml water. After proper drying of the cartridge, the HSYA-containing fraction was eluted from the column with 2 ml methanol. The eluate was evaporated to dryness under nitrogen at 40 °C. The residue was reconstituted by 200 μl MeOH-H_2O (80:20, v/v).

2.5. Preparation of the stock and standard solutions

The stock solutions of HSYA (100 μg/ml) and the internal standard (100 μg/ml) were separately prepared in methanol. Standard solutions of HSYA at concentrations of 20 μg/ml, 10 μg/ml, 2 μg/ml, 1 μg/ml, 400 ng/ml, 200 ng/ml, 100 ng/ml, 40 ng/ml and 20 ng/ml were prepared by serial dilution of HSYA stock solution with methanol. A solution containing 100 ng/ml of puerarin was also obtained by dilution of the internal standard stock solution with methanol. All standard solutions were kept at −20 °C.

2.6. Preparation of calibration curves and quality control samples

The calibration curves of HSYA were prepared at the concentration levels of 1, 2, 5 ng/ml, 10, 20, 50 ng/ml, 100, 500, and 1000 ng/ml by spiking appropriate amount of the standard solution in 1 ml blank human plasma. Three levels of HSYA quality control samples were prepared in blank human plasma at the nominal concentrations of 1, 50 and 1000 ng/ml. All samples were stored at −20 °C until analysis.

2.7. Method validation

A thorough and complete method validation of HSYA in human plasma was done following the USFDA guidelines [10]. The method was validated for selectivity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability.

Table 1
The MS parameters for analytes.

Parameter	Value
Scan type	MRM
Ion polarity	Negative
Fragmentor voltage (V)	200 (HSYA) and 160 (IS)
Nebulizer pressure (psi)	50
Drying gas temperature (°C)	350
Dry gas flow (L/min)	10
Dwell time per transition (ms)	200
Resolution	Unit
Collision energy (eV)	24 (HSYA) and 20 (IS)
Ion transition for HSYA (m/z)	611.19 → 491.19
Ion transition for Puerarin (m/z)	415.19 → 295.10

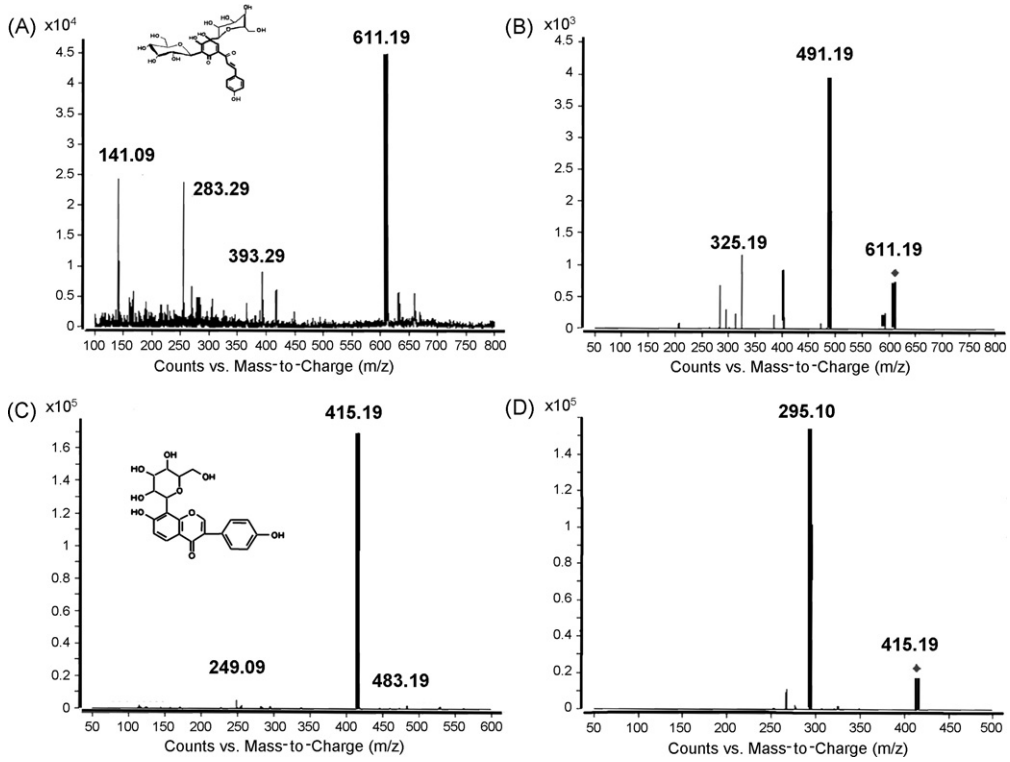

Fig. 2. Full-scan negative product ion mass spectra of (A) precursor ion of HSYA; (B) product ion of HSYA; (C) precursor ion of IS and (D) product ion of IS.

The selectivity towards endogenous and exogenous plasma matrix components was assessed in six different batches of human plasma samples by analyzing blank and spiked samples at LLOQ level. It was performed in two sets, in the first set, samples were extracted and directly injected for LC–MS/MS detection and in the second set, blank plasma samples spiked with LLOQ working solution of HSYA were extracted and analyzed. The second set was also used for sensitivity determination.

The linearity of the method was determined by analysis of standard plots associated with a nine-point calibration curve. The linearity curve containing nine non-zero concentrations was analyzed. Best-fit calibration curve of peak area ratio versus concentration was drawn. The concentrations of the analytes were calculated from the simple linear equation using regression analysis of spiked plasma standards with reciprocate of the drug concentration as a weighting factor (1/concentration, i.e. 1/x).

Inter-batch and intra-batch accuracy and precision was evaluated at three QC levels. Mean and standard deviation (S.D.) were obtained for calculated drug concentration at each level. Accuracy and precision were evaluated in terms of relative error (RE) and %CV, respectively.

Recovery presents the extraction efficiency of a method, which was performed at three QC levels. The recoveries were evaluated by comparing peak area of extracted samples to that of aqueous samples (QC working solution spiked in mobile phase).

The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended or other interfering substances in the samples. It was evaluated by comparing the peak area of the analytes dissolved in the blank plasma sample's reconstituted solution (the final solution of the blank plasma after extraction and reconstitution) with that of the analytes dissolved in the mobile phase. Three different concentration levels of HSYA (1, 50 and 1000 ng/ml) were evaluated by analyzing five samples at each level. The blank plasma used in this study was from five different batches of the blank plasma. If the peak area ratio is less than 85% or more than 115%, a matrix effect is implied.

Stability experiments were performed to evaluate the analyte stability in plasma samples under different conditions. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (−20 °C) and to three freeze-thaw stability studies. All stability studies were conducted at three concentration levels (1, 50 and 1000 ng/ml as low, middle and high values) with three determinations for each.

2.8. Application for pharmacokinetic study

The validated method was applied to evaluate the pharmacokinetics of HSYA in healthy volunteers. Twelve healthy Chinese volunteers were recruited into the study. The protocol and associated informed consent statements were reviewed and approved by the Committee on Human Rights Related to Human Experimentation, Xijing Hospital and the informed consent statements were signed by the volunteers. Safflower oral solution (containing 140 mg of HSYA) was administrated as a single dose after fasting overnight. Blood samples were collected at the predetermined intervals following administration of safflower oral solution [0,0.75, 0.5, 1, 1.25, 1.5, 2.0, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 16 and 24 h]. The samples were transferred to heparinized tube and centrifuged at 4000 *g* for 10 min. Plasma was collected and stored at −80 °C until analysis.

The pharmacokinetic parameters of HSYA were estimated using noncompartmental method. The actual blood sampling times were used, and the maximum plasma concentration (C_{max}) and the time to C_{max} (T_{max}) were determined by inspection of the individual plasma concentration–time profiles of the drug. The area under the plasma concentration–time curve from time zero to the last measurable concentration (AUC_{0-t}) was calculated using the linear trapezoidal rule. The elimination rate constant (k_e) was estimated

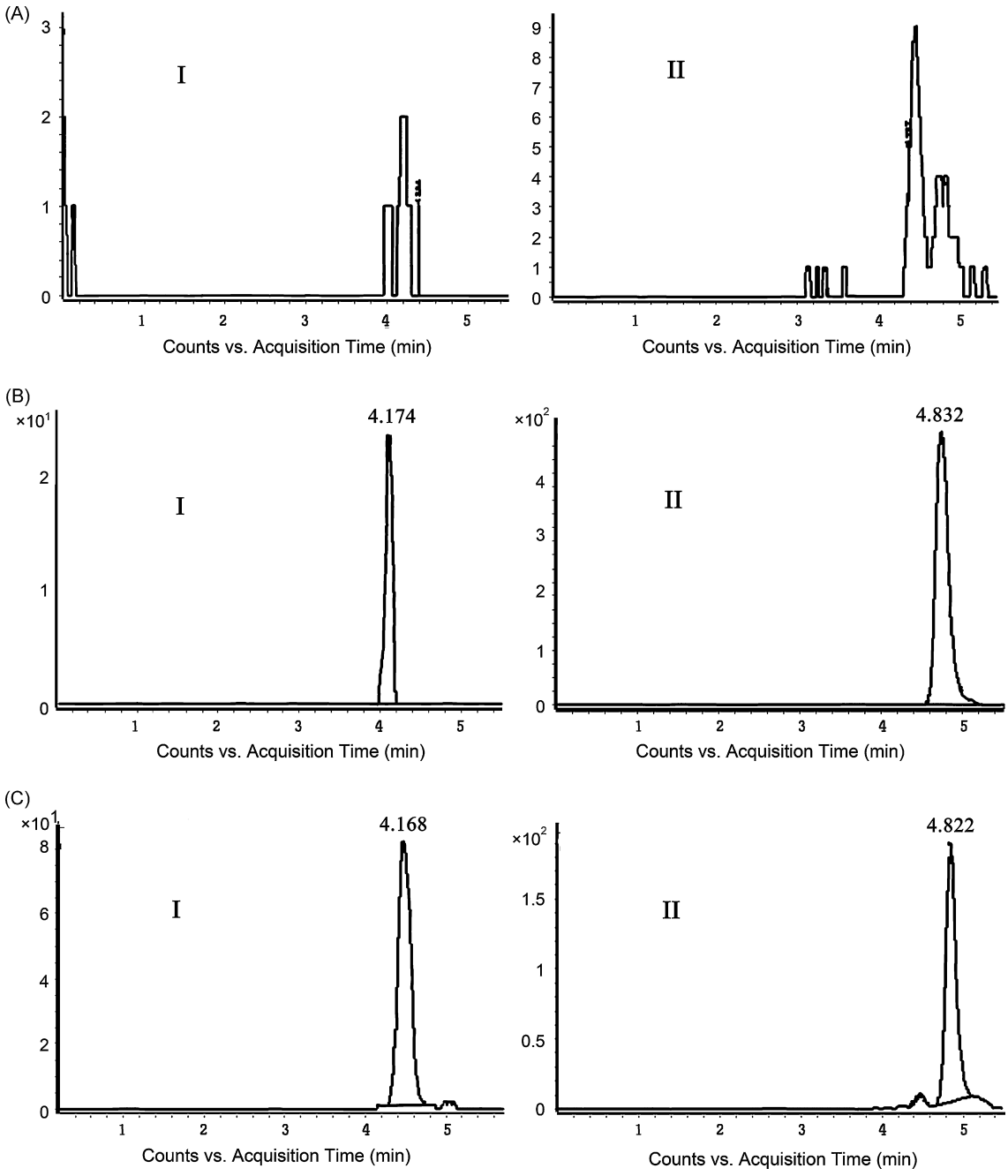

Fig. 3. Typical chromatograms of (A) blank plasma; (B) LLOQ for HSYA (1 ng/ml) in plasma an IS; (C) plasma obtained from a volunteer at 8 h after oral administration of safflower oral solution containing 140 mg of HSYA (I: HSYA, II: IS).

from the least-squares regression slope of the terminal plasma concentration. The AUC from 0 to infinity (AUC_∞) was calculated as the $AUC_\infty = AUC_{0-t} + C_t/k_e$ (C_t is the last plasma concentration measured). The half-life ($t_{1/2}$) was calculated as $\ln 2/k_e$.

3. Results and discussion

3.1. Method development

3.1.1. Selection of IS

It was difficult to *find* a compound that could ideally mirror the analyte to serve as a suitable IS. Several compounds were investigated such as rutin, quercetin and so on, but they have poor peak shape under the condition and finally puerarin (Fig. 1(B)) belonging to the same class of flavanoids was found most appropriate for the present purpose. The behavior of puerarin's retention time is similar to that of HSYA. Clean chromatographs were obtained and no significant direct interference in the MRM channels at the relevant retention times was observed.

3.1.2. Sample pre-treatment

Different sample pre-treatment methods were investigated in the present study, such as protein precipitation and liquid–liquid extraction with various organic solvents and their mixtures. However, the extraction recoveries were either low or non-reproducible (data not shown). Subsequently, SPE was investigated as samples pre-treatment technique. Following optimization of conditioning, a

Table 2
Intra- and inter-batch precision and accuracy for determination of HSYA in human plasma.

Spiked concentration of HSYA (ng/ml)	Intra-batch precision and accuracy ($n = 5$)			Inter-batch precision and accuracy ($n = 3$)		
	Measured (ng/ml)	RE (%)	CV (%)	Measured (ng/ml)	RE (%)	CV (%)
1	1.12 ± 0.03	11.81	5.81	1.04 ± 0.14	4.14	13.51
50	51.11 ± 1.35	2.23	2.84	52.46 ± 2.01	4.94	3.84
1000	1089.35 ± 33.28	8.04	3.06	1062.56 ± 46.01	6.32	4.33

Cleanert ODS cartridge (500 mg/3 ml) was selected in this assay. The SPEs pre-conditioned (2 ml methanol followed by 2 ml water) were washed with 1 ml water followed by 2 ml methanol. The eluate was subjected for drying and reconstituted to increase the sensitivity. No interference was observed from any endogenous or exogenous plasma matrix.

3.1.3. Liquid chromatography

Chromatographic analysis of the analyte and IS was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid, along with altered flow rates (in the range of 0.2–0.6 ml/min) were tested to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. It was found that a mixture of methanol 5 mM ammonium acetate (80:20, v/v) could achieve this purpose and this was finally used as mobile phase. A flow rate of 0.4 ml/min permitted a run time of 5.5 min.

3.1.4. Mass spectrometry

HSYA can be ionized under both positive and negative ionization conditions. Negative ionization mode gave more relative intense signals over positive ionization mode in MS and MS/MS data for HSYA. The product ion mass spectra of HSYA and the IS are shown in Fig. 2. $[M-H]^-$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain ion spectra. The results also showed that the most sensitive mass transition was m/z 611.19 → 491.19 for HSYA, and m/z 415.19 → 295.10 for IS. The MRM state file parameters presented in Table 1 are the optimized values for the sensitivity and selectivity required for HSYA.

3.2. Selectivity and sensitivity (LLOQ)

The selectivity of the method towards endogenous plasma matrix was evaluated in six different batches of human plasma. Fig. 3 shows the typical chromatograms of blank plasma, spiked plasma sample with HSYA and the IS, and the plasma sample from a volunteer after oral administration. The retention times of HSYA and IS were 4.7 and 4.2 min, respectively. No significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of HSYA or the IS.

The LLOQ is defined as the lowest concentration of the calibration curve yielding accuracy of ±20% and precision of ≤20%. For this method, the LLOQ of HSYA was established at 1 ng/ml with a CV of 9.2% and an accuracy of 85.8%.

3.3. Linearity, accuracy and precision

The calibration curves were based on triplicate analyses of each HSYA concentration. A linear correlation was found over the entire studied range 1–1000 ng/ml. The mean equation of the calibration curve obtained from three batches in method validation was $f = -0.027 + 0.008 \times C$, ($r^2 = 0.9995$) for HSYA, where f represents the HSYA peak area to the IS peak area ratio and C represents the HSYA concentration. A weighing factor of 1/concentration ($1/x$) was chosen to achieve homogeneity of variance.

The intra- and inter-batch precision and accuracy of the assay assessed by running a single batch of samples containing a calibration curve and five replicates at each QC level. The precision was calculated using one-way ANOVA. The results, summarized in Table 2, demonstrate that the precision and accuracy values are within the acceptable range and the method is accurate and precise.

3.4. Recovery and matrix effect

The extraction recovery of HSYA was calculated by analyzing five replicates at 1, 50 and 1000 ng/ml. The extraction recoveries of the assay were 79.42 ± 3.40, 81.70 ± 4.97 and 84.12 ± 5.87% for the low, middle and high concentration levels, respectively.

To study the effect of matrix on analyte quantification with respect to consistency in signal suppression, matrix effect was checked with five different lots of heparinized plasma. Three replicates each of three concentration levels (1, 50 and 1000 ng/ml) were prepared from different lots of plasma. The results were 89.12 ± 5.83, 92.33 ± 3.54 and 93.75 ± 4.14 for the low, middle and high concentration levels, respectively. In this study, there was no matrix effect of the analytes.

3.5. Stability

The stability results are summarized in Table 3. The data indicated that HSYA was stable under the conditions evaluated, reflecting actual sample handling and analysis. Stability of the QC samples after 24 h at room temperature and after the three freeze and thaw cycles, and post-preparative stability of the processed samples after 24 h was acceptable. HSYA in plasma at −20 °C was stable for 1 month. The RSD of the standard solution long-term freezer stability for 30 days using the concentration at 100 ng/ml was 3.72%.

3.6. Pharmacokinetic studies

The method was successfully applied to determine the plasma concentration of HSYA up to 24 h after oral administration of

Table 3
Short-term, post-preparative, and freeze-thaw stability of HSYA.

Spiked concentration of HSYA (ng/ml)	Mean of percentage remaining (%)		
	1	50	1000
Short-term (24 h, room temperature, $n = 5$)	98.53	99.45	99.14
Post-preparative stability (24 h, room temperature, $n = 5$)	100.04	98.82	97.46
Freeze and thaw stability (three cycles, −20 °C, room temperature, $n = 5$)	101.47	100.75	99.68

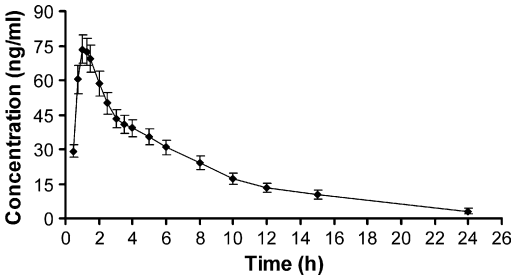

Fig. 4. Mean plasma concentration–time curve of HSYA after administration of safflower oral solution containing 140 mg HSYA to 12 healthy volunteers. (Vertical bars indicate mean standard error.).

safflower oral solution to the healthy volunteers. Mean plasma concentration–time profile is presented in Fig. 4. In the present study, $t_{1/2}$ ranged from 2.6 to 3.5 h, with a mean value of 2.9 h. The C_{max} was 73.5 ± 8.7 ng/ml. T_{max} was 1 h. $AUC_{0-24\,h}$ and AUC_{∞} were calculated and the ratio $AUC_{0-24\,h}/AUC_{\infty}$ was higher than 85% for all volunteers.

4. Conclusion

A sensitive, specific and accurate method is described for the quantification of HSYA in human plasma by LC–MS/MS in negative electrospray ionization mode using MRM and fully validated according to commonly accepted criteria. The desired sensitivity of HSYA was achieved with an LLOQ of 1 ng/ml, and the short run time was suitable for analysis of the large batches of samples. The method has been successfully used for pharmacokinetic studies.

Acknowledgments

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