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Simultaneous estimation of mangiferin and four secoiridoid glycosides in rat plasma using liquid chromatography tandem mass spectrometry and its application to pharmacokinetic study of herbal preparation☆

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Abstract

Extracts from *Swertia chirata* (family *Gentianaceae*) have antidiabetics and antioxidant activity, largely attributed to the flavonoids and secoiridoids, which are a major class of functional components in methanolic extracts from aerial part of plants. In order to facilitate analysis of systemic exposure to *S. chirata* derived products in animals, we developed a liquid chromatography tandem mass spectrometry (LC–MS/MS) based method that is capable of routinely monitoring plasma levels of flavonoids and secoiridoids. An LC–MS/MS-based method has been developed for the simultaneous estimation of two bioactive markers, mangiferin and amarogentin along with three other components, amaroswerin, sweroside and swertiamarin in rat plasma. All the analytes including the internal standard (kutkoside) were chromatographed on RP-18 column (250 mm × 4 mm i.d., 5 μm.) coupled with guard column using acetonitrile: 0.5 mM ammonium acetate buffer, pH ∼3.0 as mobile phase at a flow rate of 1 ml/min in gradient mode. The final flow to source was splitted in 1:1 ratio. The detection of the analytes was performed on API 4000 LC–MS/MS system in the multiple reaction-monitoring (MRM) mode. The quantitation for analytes other than the pure markers was based on relative concentration. The method was validated in terms of establishing linearity, specificity, sensitivity, recovery, accuracy and precision (Intra- and Inter-day), freeze-thaw stability, peltier stability, dry residue stability and long-term stability. The recoveries from spiked control samples were >90% for all analytes and internal standard except mangiferin where recovery was >60%. Intra- and inter-day accuracy and precision of the validated method were within the acceptable limits of <15% at low and <10% at other concentrations. The quantitation method was successfully applied to generate pharmacokinetic (PK) profile of markers as well as to detect other components in plasma after intravenous dose administration of herbal preparation in male Sprague-Dawley (SD) rats.

Keywords: LC–MS/MS; Bioanalysis; *Swertia chirata*; Mangiferin and secoiridoid glycosides

1. Introduction

The plant *Swertia chirata* (family *Gentianaceae*) commonly known as 'Chirata', is well known for its multifarious therapeutic value and widely used in Indian System of medicine as a crude drug. It is used as an antimalarial, a bitter stomachic, anthelmintic, and as a remedy for scanty urine, epilepsy, ulcer, bronchial asthma and certain type of mental disorder [1]. Studies on the biological activities of *S. chirata extract* reveal that this bitter plant possesses antioxidant, antidiabetic, antimicrobial, anticholinergic and chemopreventive activity [2–5].

Early studies documented the presence of flavonoids, xanthones, terpenoids, iridoid and secoiridoid glycosides in *S. chirata* plant [6]. Phytochemical investigations on bioactive methanolic extract of defatted aerial part of *S. chirata* plant led to the isolation of the mangiferin (A), a xanthone-C-glycoside and amarogentin (B), a secoiridoid-O-glycoside, while three more secoiridoid glycosides amaroswerin (C), sweroside (D) and swertiamarin (E) were structurally correlated and confirmed on the basis of liquid chromatography tandem mass spectrometry (LC–MS/MS) (Fig. 1) [7]. A chirata-based product (CT) is a

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herbal antidiabetics developed by Central Drug Research Institute (CDRI), Lucknow, from aerial part of *S. chirata*. Mangiferin and amarogentin are two bioactive markers of CT used for its standardization. In order to better understand the pharmacological and beneficial effects of chirata-based phytomedicine, it is required to perform pharmacokinetic measurement of these bioactive components. However, such studies have been complicated by the lack of sensitive methods for analysis of these components in biological matrices.

So far, procedure for separation, detection and quantitation of all of the above mentioned glycosides in plasma has not been published in contrast to methods for quantitation of single substances of this group or combinations of few of them. For the analysis of mangiferin in biological fluids, various procedures have been published using high performance liquid chromatography/ultraviolet (HPLC/UV) [8–11]. More recently, Wang et al. developed a HPLC/UV-based method for measuring mangiferin in rat plasma and urine following its oral administration [12]. These methods, however, have generally shown low sensitivity, with limit of quantitation (LOQ) ranging from ~0.48 to 0.6 μg/ml. Liquid chromatography coupled with tandem mass spectrometry was also used for analysis of free mangiferin in rat blood using microbore LC–MS/MS, providing LOQ with ~0.05 μg/ml and recovery ranging from ~37.7 to 39.8% [13]. Vipul et al. developed an LC–MS/MS-based bioanalytical method for determination of two iridoid glycoside (picroside-I and kutkoside) in rabbit plasma, where amarogentin was used as internal standard (IS) and this method required lengthy sample clean up step using solid phase extraction [14].

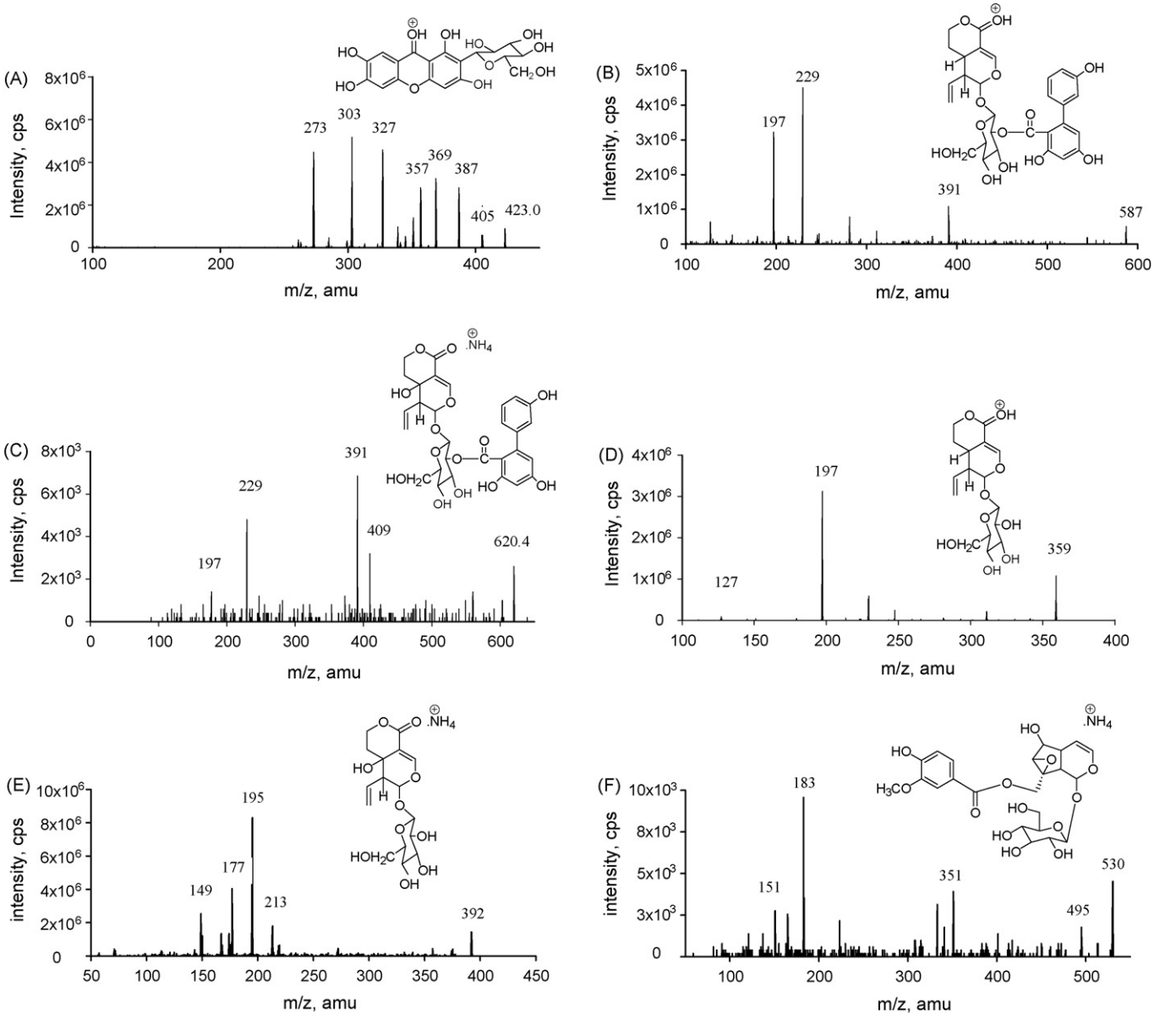

Fig. 1. MS–MS spectra of (A) mangiferin, (B) amarogentin, (C) amaroswerin, (D) sweroside (E) swertiamarin and (IS) Kutkoside showing prominent precursor to product ion transitions.

To our knowledge, no prior reports have described a sensitive LC–MS/MS-based method for determining these xanthone- and secoiridoid glycosides from plasma. Furthermore, although pharmacokinetic evaluation of phytomedicine based on maximum number of markers is better to ensure clinical and pharmacological repeatability, it is really cumbersome and always not possible to obtain pure authentic markers from any herbal preparation. Accordingly, we have included three more components in assay method besides two standard markers of herbal preparation on the basis of their earlier reported LC–MS/MS characterization. These components could be monitored to generate stability profile in biomatrices as well as concentration independent pharmacokinetic parameter in preclinical studies. However, such application has not been mentioned in previous reports describing the bioanalytical assay with application in pharmacokinetic study of any phytomedicine.

We herein sought to develop an LC–MS/MS-based assay capable of sensitively detecting and quantifying plasma chirata xanthone- and secoiridoid glycosides, for use in pharmacokinetic measurement of chirata-based herbal preparation from rat plasma. The method was validated in two parts: first one with pure markers (mangiferin and amarogentin); and second one with herbal preparation, where in, three components amaroswerin, sweroside and swertiamarin were monitored. The method was successfully applied in pharmacokinetic evaluation of chirata-based phytomedicine in rat following intravenous administration.

2. Experimental

2.1. Reagents and standards

Pure reference standards (purity >99%) of mangiferin and amarogentin were procured from the Medicinal and Process Chemistry Division of CDRI, Lucknow, India. Acetonitrile (HPLC grade) and glacial acetic acid (HPLC grade) were purchased from Thomas Baker, Mumbai, India. Dimethyl sulphoxide (DMSO) was obtained from Ranbaxy Laboratories Ltd., Punjab, India. Ammonium acetate (GR-grade) was procured from E Merck (India) Ltd., Mumbai. Ultra pure water of 18.2 MΩ cm was obtained from MilliQ PLUS purification system (Millipore, USA). IS kutkoside (purity >99%) was obtained in house. Blank rat plasma was collected from healthy, drug-free Sprague-Dawley (SD) at the Laboratory Animal Center of CDRI, Lucknow, India. Plasma was obtained by centrifuging the heparinized blood at 2000 rpm for 15 min.

For pharmacokinetics study, young and healthy male SD rats weighing 250 ± 50 g were obtained from the Laboratory Animal Center, CDRI and housed in appropriate stainless steel cages in standard laboratory conditions with regular 12 h day–night cycle in well-ventilated room with an average temperature of 25–28 °C and relative humidity of 40–60%. Standard pelleted laboratory chow (Goldmohar Laboratory Animal Feeds, Chandigarh, India) and water allowed *ad libitum* to rats. All ethical guidelines for maintenance and experimental studies with SD rats were followed.

2.2. Plant material and herbal preparation

S. chirata (Roxb ex flam) Karsh (*Gentianaceae*) grows abundantly at an altitude of 1220–3050 m in sub Himalayan region. The plant was collected in September–October by Botany Division, CDRI, Lucknow, India. Capsules filled with powdered herbal preparations (CT) were provided by Clinical and Experimental Pharmacology Division, CDRI, Lucknow, India.

2.3. Stock solution, calibration standards and quality control samples

Stock standard solutions (1 mg/ml) of component A, B and IS were prepared in acetonitrile (0.2%, v/v DMSO) and were used to prepare working standard solutions. Analytical standards for A and B were prepared in mobile phase (1:1, v/v) over a concentration range of 1.56–200 ng/ml and 0.156–20 ng/ml, respectively by serial dilution, and same concentration range for calibration curve were prepared in blank normal rat plasma. Quality control (QC) samples at four different concentration levels (3.13, 6.25, 25, 100 ng/ml for A and 0.313, 0.625, 2.5 and 10 ng/ml for B as low 1, low 2, medium and high, respectively) were prepared in three sets independent of the calibration standards. During analysis these QC samples were spaced after every six to seven unknown samples.

Stock solution of standard herbal preparation (1 mg/ml), that consists of varying percentage of components C, D and E (where, peak area ratio of each analytes to IS was considered equal to 1000 arbitrary unit) was prepared in water with 10% acetonitrile and was used to prepare working stock solutions. Analytical standards were prepared in mobile phase (1:1) over a range of 0.156–20 arbitrary unit for each analytes by serial dilution and calibration standards were prepared in blank normal rat plasma over a same concentration range. QC samples at four different concentration levels (0.313, 0.625, 2.5 and 10 arbitrary unit as low 1, low 2, medium and high respectively) were prepared in three sets independent of the calibration standards.

2.4. Sample preparation

Sample preparation involved simple protein precipitation method using acetonitrile (1:1, v/v). The processing volume of plasma was fixed as 100 μl. The IS solution was spiked into such aliquots as to give a final concentration of 4 ng/ml of kutkoside and vortex mixed (Type 37600 mixer, Thermolyne, USA) prior to the addition of the extraction solvent. After mixing thoroughly with vortex mixer for 1 min and centrifugation (10,000 rpm for 5 min), the supernatant (20 μl) was injected onto the LC–MS/MS system. For determination of matrix effect control drug free plasma was protein precipitated using acetonitrile as described above and final supernatant was evaporated to dryness. Dry extracts were dissolved using analytes and IS standard solutions that represent 100% recovery. Ion suppression was determined by comparing the analytical response of these samples with that of standard solutions.

2.5. Chromatographic condition

A Perkin-Elmer Series 200 pump (Perkin-Elmer, Shelton, CT, USA) consisting of flow control valve, vacuum degasser (Series 200 vacuum degasser) operated in gradient mode to deliver the mobile phase at flow rate of 1.0 ml/min. The chromatographic system consisted of RP-18 column (250 mm × 4 mm i.d., 5 μm) (LiChroCART®, Merck, Germany) coupled with a guard column of same material (Applied Biosystems, Toronto, Canada), and solvent A: acetonitrile and solvent B: ammonium acetate buffer, 0.5 mM, pH 3.0 as mobile phase. The linear gradient programme that gave the optimal sensitivity was following: linear increase from 10% solvent A to 50% over 4 min, maintained isocratically over 2 min, decreased linearly to 10% solvent A over 2 min and maintained at that levels for further 4 min. In total run time of 12 min, all analytes eluted between 6–8 min. The total effluent from the column was diverted to waste for initial 0–5 min and from 9 to 12 min of total run time using a flow diverter assembly (Valco valve, VICI, Valco Instrument Company Inc., USA) to avoid unnecessary contamination to mass spectrometer and final flow to source was split such that half was injected onto the electrospray ionization (ESI) source. The analysis was carried out at ambient temperature and the pressure of the chromatographic system was 2400–2500 psi.

2.6. Mass spectrometric condition

Mass spectrometric detection was performed on API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) equipped with a Turbo V IonSpray ionization source operating in positive ESI mode. In mass spectrometer, zero air was used as nebulizing gas (GS 1) and turbo gas (GS 2) while nitrogen as curtain gas (CUR) and collision activated dissociation (CAD) gas and these gases were constantly supplied from a gas generator (Peak Scientific, USA). MS and MS/MS condition for pure standard of marker A, B and IS was optimized by continuous infusion at 5 μl/min using syringe pump (Model '11', Harvard apparatus, Inc., Holliston, MA, USA) while for component C, D and E known concentration of CT subjected to LC/ESI-MS analysis. Information dependent acquisition (IDA) method was used to generate selective pattern of component C, D and E from herbal preparation CT. MS/MS analysis of all analytes were performed using nitrogen as CAD gas. The most abundant product ion of each component was selected to build multiple reaction-monitoring (MRM) method. The transitions monitored were *m/z* 423 > 357, 587 > 229, 620.4 > 391, 359 > 197, 392 > 195 and 530 > 183 for components A–E and IS, respectively. Nebulizing gas, turbo gas (GS 2), curtain gas (CUR), capillary voltage and temperature were set to 30, 45, 15 psi, 5500 kV and 400 °C, respectively. The mass spectrometer was operated in unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 200 ms per MRM channel. All data were acquired within 5–9 min using Analyst 1.4 software (Applied Biosystems/MDS SCIEX) with delay time setting of 5 min.

2.7. Application to pharmacokinetic study

The method was successfully applied to generate the plasma concentration time profile of pure markers (A and B) as well as to detect other three components (C, D and E) in plasma following i.v. administration at 5 mg/kg dose of herbal preparation CT in male SD rats. Intravenous formulation was prepared in solution form by dissolving accurately weighed amount of powdered herbal preparation (from capsules) in normal saline (0.9%, w/v sodium chloride). The formulations were sterilized by filtration (using 0.45 μm filter) before use. Intravenous dose (0.25 ml/250 g rat) was administered using a 1.0 ml tuberculin syringe (fitted with 26G needle) via caudal vein at 5 mg/kg of herbal preparation. The pilot pharmacokinetic (PK) study was carried out by sparse sampling approach wherein blood samples were collected from single rats per time point ($N = 1$). Two samples were collected from each rat in the study group, first by cardiac puncture (~0.25 ml) followed by terminal sampling from inferior vena cava. Blood samples were collected at 5, 10, 20, 30, 45 min, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, 24 and 48 h and separated plasma was stored at −50 °C pending analysis.

3. Results and discussion

3.1. Mass spectrometry

In positive ion mode all analytes give protonated ion at *m/z* $[M+H]^+$ and sodium adduct at *m/z* $[M+Na]^+$ and later was observed as a base peak in case of secoiridoid glycosides. However, the division of signal between sodium and protonated ions resulted in compromised sensitivity. The division of signal between sodium, potassium and protonated ions resulted in compromised sensitivity. Hence, possibility of formation of intense protonated or ammonium adduct was explored, using ammonium acetate buffer in combination with acetonitrile. In natural product analysis, the most common additives used with the application in ESI are acetic acid, formic acid, ammonium acetate and ammonium formate. Trifluoroacetic acid has also been used in some studies despite the fact that in many cases it is found to suppress the ionization owing to ion pairing and surface tension effects.

In order to find most sensitive ionization mode for the components studied, +ESI and −ESI were tested with various combination of mobile phase, i.e. acetonitrile and water/ammonium acetate buffer (0.5 mM)/glacial acetic acid (0.1%) in positive and negative ionization mode.

In positive LC/ESI-MS experiment, the components studies showed a very high tendency to form sodium adduct in acetonitrile:water. The sodium adduct $[M+Na]^+$ was the main peak in the spectra of all components, which is expected for these phenolic glycosides. It was observed that the signal intensity for $[M+H]^+$ ions in positive ion ESI mode was 2–10-fold higher for all components in analyses using acetonitrile:ammonium acetate buffer (0.5 mM), versus experiments run with the corresponding electrolyte free mobile phase.

In negative ion ESI mode experiments, the peak due to the deprotonated molecule $[M-H]^-$ was seen in all spectra and was

clearly main peak for all components except D and E with electrolyte free mobile phase. The signal intensity for deprotonated ion at *m/z* $[M-H]^-$ of all analytes in mobile phase containing ammonium acetate buffer 0.5 mM, was lower than experiments run with the corresponding electrolyte free mobile phase. The use of mobile phase containing 0.1% (v/v) glacial acetic acid yielded signal intensity of *m/z* $[M-H]^-$ ion with 6.6–7-fold higher for component A, while for other analytes, intensity of *m/z* $[M-H]^-$ ion was low to those obtained in experiments run with electrolyte free mobile phase.

The negative ion ESI mode was shown to result in about 2–10 times lower signal-to-noise ratios than the positive ion mode, when the most sensitive eluent systems for each components were compared. The only exception to this was component A, for which negative ion mode resulted more sensitive analytes responses than positive ion modes. Thus, it was decided to utilize positive ion mode for detection and quantitation of $[M+H]^+$ and/or $[M+NH_4]^+$ ions, which on fragmentation gave prominent and stable product ions. The optimized declustering potentials for the protonated $[M+H]^+$/ammonium adducts $[M+NH_4]^+$ of component A–E and IS were found to be 62, 90, 60, 80, 46 and 50 V, respectively.

With optimized MS conditions, MRM mode was explored for all analytes. Initially product ions were generated through fragmentation of the molecular ions by CAD, using nitrogen as collision gas. The production spectra of components in positive ion mode are given in Fig. 1. In brief, the product ion spectrum of the $[M+H]^+$ ion at *m/z* 423 (component A) showed fragment ion at *m/z* 405 and 387 corresponding to subsequent loss of two water units, $[M+H-18]^+$ and $[M+H-36]^+$, respectively, followed by loss of neutral as formaldehyde [–HCHO] yielded fragment ion at *m/z* 357. Spectrum reveals that fragment ion at *m/z* 303 corresponding to loss of 120 mass units due to fragmentation in the C-glycosidic unit was less abundant in positive ion mode. The MS/MS spectra of the $[M+H]^+$ ion at *m/z* 587 (Component B) and $[M+NH_4]^+$ ion at *m/z* 620 (component C) yielded common prominent fragment ion *m/z* 391 corresponding to loss of iridoid moiety. MS/MS spectra of protonated and ammoniated ion at *m/z* 359 and 397 for component D and component E respectively, showed characteristic fragment at *m/z* 197 and *m/z* 195 corresponding to mono-hydroxylated iridoid moiety. Utilizing this information, two or three intense product ions were selected to build an MRM method for quantitation and collision energies (CE) were optimized for different transitions by direct infusion.

3.2. Liquid chromatography

Although MS was used as the detector, the method development was focused on chromatographic separation of herbal components that considered advantageous to maintain the dynamic range and to minimize the risk of ion suppression in ESI. Acetonitrile rather than methanol was chosen as the organic modifier because of its slightly higher resolving power. Moderately high acidic ammonium acetate buffer 0.5 mM, pH ~3, was required to achieve acceptable peak width and shapes.

An endcapped C_{18} column (250 mm × 4 mm i.d., 5 μm) (LiChroCART®, Merck, Germany) coupled with a guard column of same material (Applied Biosystems, Toronto, Canada) with acetonitrile:ammonium acetate buffer in gradient mode was applied in final LC method. Within the total analysis time of 12.0 min, all components eluted in 6.0–8.0 min.

A column with narrow internal diameter (Luna C_{18}, 100 mm × 2 mm i.d, particle size 3 μm, Phenomenex, CA, USA) was tested in the development stage with the solvent flow rate decreased to 100 μl/min. Despite the slightly improved resolution, proper retention was not achieved for component A. Additionally, the analysis time expanded to 20 min, mainly due to the equilibration step after the gradient, and irreproducibility was still observed in the retention times.

3.3. Optimization of LC–MS/MS condition

Final MRM transitions were selected on the basis of signal to noise ratio (*S/N*) ratio with on-column injection analysis. Nebulizing gas (GS 1), turbo gas (GS 2), curtain gas and temperature were set to 30, 45, 15 psi and 400 °C, respectively. The transitions selected were *m/z* 423 > 357, 587 > 229, 620.4 > 391, 359 > 197, 392 > 195 and 530 > 183 for components A–E and IS, respectively. Proposed structures of prominent fragment ions selected for final MRM method are given in Fig. 2. The selection of kutkoside, a secoiridoid glycoside as IS was based on its structural similarity with most of the analytes. Hence, kutkoside was expected to behave closely in terms of ionization giving better results for linearity and quantitation. The corresponding final MRM conditions for A–E and IS are summarized in Table 1.

3.4. Sample clean up

The next step was to develop simple and efficient sample clean up devoid of matrix suppression and interference from

Table 1
Optimized MRM conditions for components A–E and IS

Code	Analytes	Parent ion	Product ion	Declustering potential (V)	Collision energy (eV)
A	Mangiferin	423$[M+H]^+$	357	62	20
B	Amarogentin	587$[M+H]^+$	229	90	40
C	Amaroswerin	620.4$[M+NH_4]^+$	391	60	16
D	Sweroside	359$[M+H]^+$	197	80	32
E	Swertiamarin	392$[M+NH_4]^+$	195	46	19
IS	Kutkoside	530$[M+NH_4]^+$	183	50	19

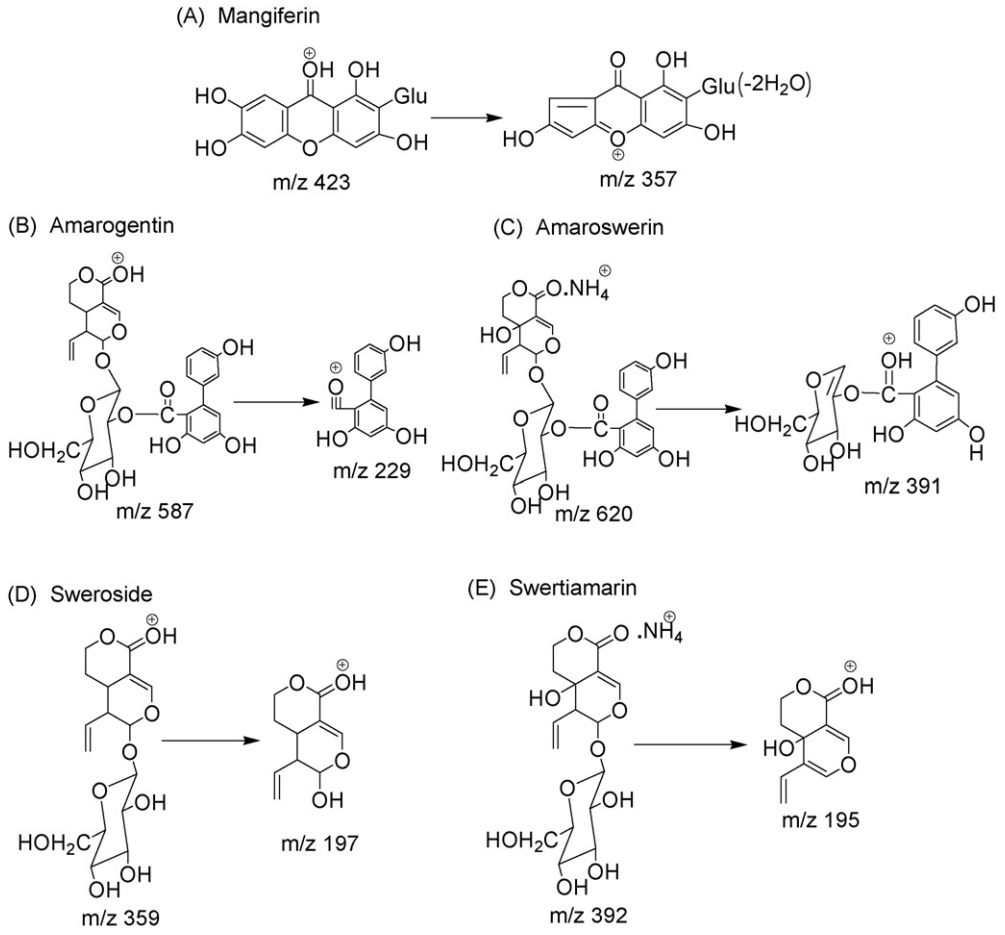

Fig. 2. Proposed structure of prominent fragment ions used in MRM transition of (A) mangiferin, (B) amarogentin, (C) amaroswerin, (D) sweroside (E) swertiamarin and (IS) kutkoside.

endogenous plasma components for estimation of the analytes in rat plasma. Liquid–liquid extraction (LLE) using ether and different combinations of hexane and ethyl acetate (90–10%, v/v), *n*-hexane and IPA (2–5%, v/v) was tried but none of these was found suitable to give good and consistent recovery for all analytes. Finally, protein precipitation using acetonitrile was tried and found suitable to give optimum recovery for all analytes. Although protein precipitation yielded strong matrix effect than other sample clean up methods like LLE and solid phase extraction (SPE), has an advantage of simple and fast techniques. The matrix issue seems more complex when analyzing multiple analytes from biosample in a single analytical run in quantitative LC–MS/MS, but this could be minimized with initial optimization of chromatographic condition to elute matrix and analytes distinctly. Thus, we investigated %matrix interference on different chromatographic elution of analytes during initial optimization of chromatographic condition. It was observed that matrix suppression eliminated completely with present optimized gradient condition.

3.5. Method validation

Accuracy, precision, selectivity, sensitivity, linearity and stability were measured and used as the parameter to assess the assay performance. The peak area ratios of analytes to IS in rat plasma were linear over the concentration range 3.13–200 ng/ml for A and 0.156–20 ng/ml for B and 0.156–20 arbitrary unit for component C, D and E. The calibration model was selected based on the analysis of the data by linear regression with and without intercepts ($y = mx + c$ and $y = mx$) and weighting factors ($1/x$, $1/x^2$ and $1/\log x$). The best fit for the calibration curve could be achieved by a linear equation of $y = mx + c$ and a $1/x^2$-weighting factor for all components. The correlation coefficient (R) for component A and B were above 0.998 over the concentration range used.

LC–MS/MS analysis of the blank plasma samples showed no interference with the quantification of component A–E and the IS. The specificity of the method was established with pooled and individual plasma samples from eight different sources. Representative overlaid chromatogram of extracted blank rat plasma and blank plasma fortified with analytical standards and IS, demonstrating the specificity and selectivity of the method are shown in Fig. 3. The retention times of all the analytes and the IS showed less variability with a relative standard deviation (R.S.D.) well within acceptable limits of 5%.

The limit of detection (LOD) demonstrated *S/N* of ≥3 for 1.56, 0.078 ng/ml, 0.078, 0.313 and 0.078 arbitrary unit of

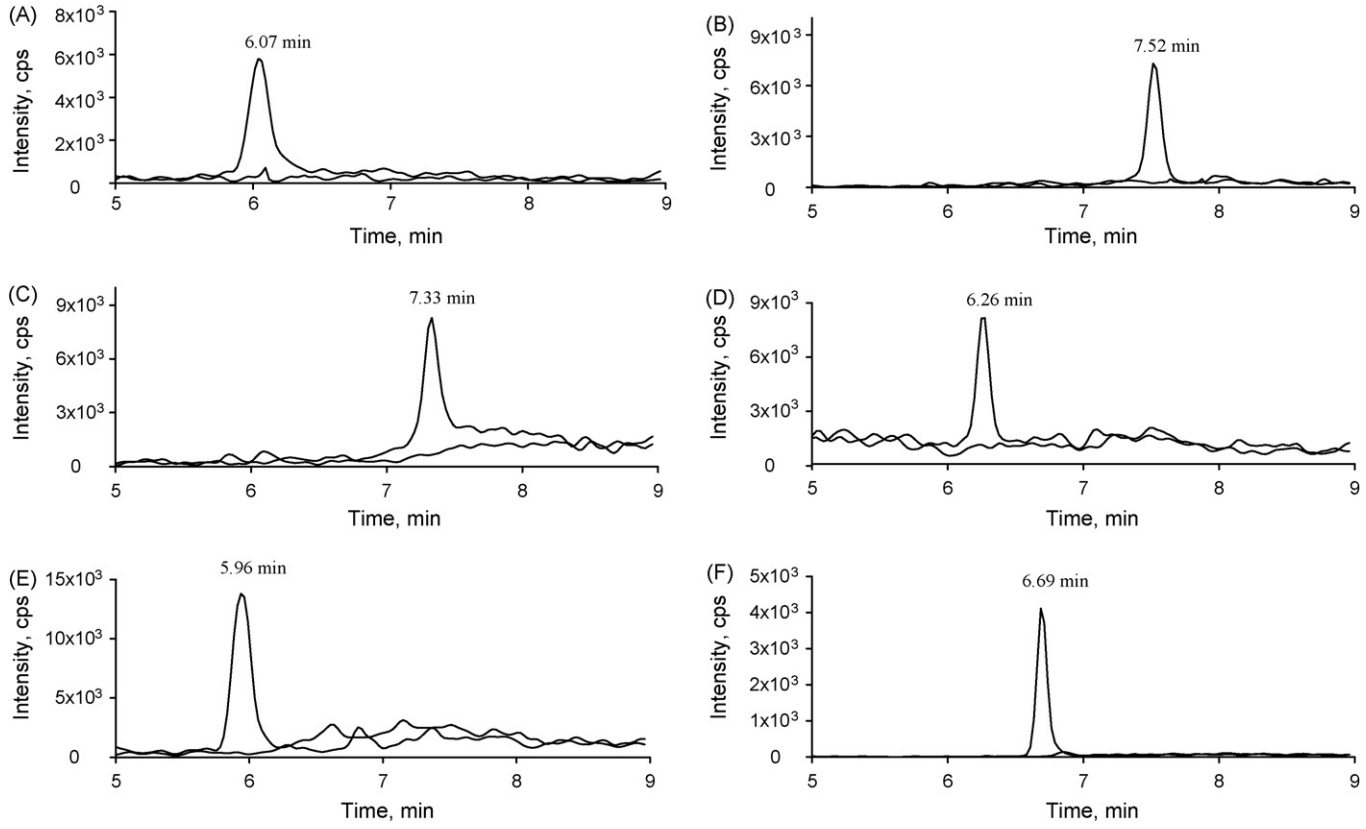

Fig. 3. Representative chromatograms of component A–E (at respective LOQ) and IS in fortified blank rat plasma overlaid with extracted blank rat plasma.

component A–E respectively. The lower limit of quantitation (LLOQ), the lowest concentration in the standard curve which can be measured with acceptable accuracy and precision for component A–E from normal rat plasma was established as 3.13, 0.156 ng/ml, 0.156, 0.625 and 0.156 arbitrary unit, respectively. The LLOQ was established with five samples independent of the standard curve.

Recovery was calculated from average peak area of processed quality control samples read against that of analytical standards as well as analytical standards fortified with extracted blank plasma. In both cases recovery was found to unchanged thus the net responses have negligible ion suppression due to matrix effect. These experiments were performed at three-concentration levels (low, medium and high) in triplicate. The average absolute recoveries for all components at three different concentrations are shown in Table 2. The average recoveries for component A–E and IS were ranged from $68 \pm 4.29\%$, $102 \pm 6.49\%$, $97.58 \pm 7.13\%$, $96.49 \pm 12.31\%$, $92.43 \pm 5.77\%$ and $85 \pm 1.23\%$ respectively.

The accuracy and precision (intra- and inter-batch) were calculated with four QC samples in triplicate for three different days and are presented in Table 3. The precision was determined by one-way ANOVA as within and between assay %R.S.D. and accuracy was expressed as %bias. The results show that the method is accurate as the bias is within acceptable limits of ±20% of the theoretical value at LLOQ and ±15% at all other concentration levels. The precision around the mean value never exceeded 15% at any of the concentrations studied.

3.5.1. Stability studies

QC samples were subjected to short term and long term storage condition (−50 °C), freeze-thaw stability, auto-injector stability and dry residue stability studies. All stability studies

Table 2
Mean recoveries of components A–E and IS in spiked rat plasma

Concentration levels[a]	(%) Absolute recovery (mean ± S.D., $n = 5$)				
	A	B	C	D	E
Low 2	69.83 ± 1.45	102.77 ± 4.82	95.37 ± 5.49	88.47 ± 8.29	89.13 ± 2.88
Medium	67.9 ± 3.99	102.12 ± 2.55	98.53 ± 1.73	100.60 ± 8.94	91.63 ± 4.37
High	66.57 ± 1.93	98.77 ± 2.15	93.77 ± 0.18	100.30 ± 2.36	90.3 ± 1.85

[a] Concentration levels (low 2, medium, high) vary according to components, where, 6.25, 25, 100 ng/ml for component A, 0.625, 2.5, 10 ng/ml for components B and 0.625, 2.5 and 10 arbitrary unit for components C, D and E represents the concentration levels low 2, medium, high, respectively.

Table 3
Accuracy (%bias) and precision (%R.S.D.) of components A–E in rat plasma

Analyte	Concentration (ng/ml)	%Bias		%R.S.D.	
		Intra-day	Inter-day	Intra-day	Inter-day
A	3.13	8.53	9.17	7.0	14.5
	6.25	−0.35	0.02	2.8	3.0
	25	−0.44	−0.35	5.1	9.4
	100	9.92	12.11	0.5	8.5
B	0.313	1.18	3.93	6.0	13.8
	0.625	1.54	1.58	5.0	9.6
	2.5	4.45	5.48	4.6	4.6
	10	−0.40	−2.16	4.9	9.2
C[a]	0.156	1.32	2.01	2.7	7.0
	0.625	−3.68	−3.57	8.6	10.5
	2.5	0.39	0.43	4.0	5.1
	10	−2.66	−2.77	3.8	3.6
D[a]	0.625	4.04	6.99	6.0	10.0
	2.5	−4.94	−5.76	5.1	10.0
	10	−1.46	−1.74	4.6	5.7
E[a]	0.156	−2.40	−7.20	1.6	13.9
	0.625	−3.86	−3.31	3.9	3.8
	2.5	−5.56	−5.13	5.6	8.0
	10	−3.32	−4.76	3.8	7.1

[a] Relative concentrations are expressed as arbitrary unit.

were carried out at two concentration levels (low and high) in triplicate.

3.5.1.1. Freeze-thaw (f-t) and long term stability. QC samples at low and high concentrations in triplicate (six sets) were prepared. One set of samples at each concentration level was analyzed immediately after spiking, which served as the reference concentration while other five sets were stored at −50 °C. Three sets were analyzed after 1, 2 and 3 f-t cycles in different run. Thawing was achieved by keeping the stored samples undisturbed at ambient temperature for 30 min. The remaining two sets of QC sample were analyzed after 15 and 30 days without any f-t cycles. The change in concentration during the f-t cycles and long term storage in rat plasma were determined by comparing the observed concentrations with the reference concentration and expressed as %deviation. The deviation observed after one, two and three f-t cycles were within the acceptable limits of ≤20% at low and ≤15% at high concentration levels for components A–E (Table 4). Also, the QC samples stored at −50 °C were analyzed after 30 days and there were no significant deviations with respect to the immediately analyzed samples (Table 5).

Table 4
Freeze-thaw (f-t) stability data for components A–E

Analytes	Concentration (ng/ml)	%Deviation from reference		
		f-t 1	f-t 2	f-t 3
A	3.13	14.70	0.78	4.68
	100	10.05	8.63	6.92
B	0.313	−13.25	−8.94	−5.38
	10	−5.75	−0.54	0.32
C[a]	0.156	6.28	0.72	−3.33
	10	−1.19	5.40	−0.24
D[a]	0.625	7.14	6.81	2.27
	10	−6.28	−7.03	−7.53
E[a]	0.156	−14.61	−10.98	−11.42
	10	−6.56	−7.99	−3.23

[a] Relative concentrations are expressed as arbitrary unit.

3.5.1.2. Peltier stability. The spiked samples at low 1 and high concentrations were processed and reconstituted at the same time. The reconstituted samples were placed in the auto injector (Tray type-peltier set at 4 °C), and one set was injected immediately and the other after 12 h. The percent deviation at two concentration levels was calculated. There was no significant difference observed between the responses of standards at time zero and after 24 h kept at auto injector at 4 °C in terms of %CV (≤5%) for all analytes, indicating sufficient stability in auto injector for completing large set of analysis (Table 5).

Table 5
Stability data for components A–E

Analytes	Concentration (ng/ml)	%Deviation			
		Dry residue stability at 4 °C	Peltier stability at 4 °C	Long term stability at −50 °C	
				15 Days	30 Days
A	3.13	3.29	2.89	6.02	8.09
	10	5.86	2.59	5.68	6.02
B	0.313	−1.80	−1.49	−1.83	0.68
	10	0.64	3.83	0.32	3.19
C[a]	0.625	−4.90	0.16	−1.53	−2.04
	10	2.48	1.90	1.29	1.83
D[a]	0.625	8.81	4.47	9.48	1.37
	10	−9.80	−0.99	−5.62	5.19
E[a]	0.625	−2.06	0.61	−0.98	2.5
	10	−10.55	1.46	1.56	2.83

[a] Relative concentrations are expressed as arbitrary unit.

3.5.1.3. Dry residue stability. QC samples at low and high concentration in triplicate were processed and one set was stored at −50 °C in glass tubes. One set of samples was analyzed immediately which served as the reference concentration. The other set was analyzed after 15 days. The change in concentration was determined by comparing the concentrations observed after 15 days with the reference concentration and was expressed as %deviation. The dry residue samples stored at −50 °C after extraction was found to be stable for over 15 days with %deviation ≤10% at all concentration levels (Table 5).

3.6. Application to pharmacokinetic study

The method described above was successfully applied to a preliminary PK study in which plasma concentration of pure markers was determined for upto 48 h after i.v. administration at 5 mg/kg dose of CT in male SD rats. The plasma concentration time profile of marker mangiferin and amarogentin are shown in Fig. 4(A), and could be traceable upto 24 h and 0.75 h, respectively. Moreover, the method successfully applied to detect other three components, i.e. amaroswerin, sweroside and swertiamarin in plasma, as shown in Fig. 4(B), and could be traceable upto 3.0, 2.0 and 3.0 h, respectively. Pharmacokinetic parameters were calculated from the plasma concentration time data by noncompartmental analysis using WinNonlin standard edition, version 1.5. The elimination half life of mangiferin and amarogentin were 1.09 and 1.23 h, respectively. Systemic exposure, clearance and volume of distribution at steady state (V_{ss}) obtained with intravenous administration were 1303.8 ng h/ml, 0.1 l/h/kg and 0.14 l/kg for mangiferin and 9.29 ng h/ml, 6.03 l/h/kg and 5.34 l/kg for amarogentin, respectively. Half life of amaroswerin, sweroside and swertiamarin were ∼0.86, 0.74 and 0.5 h in rats.

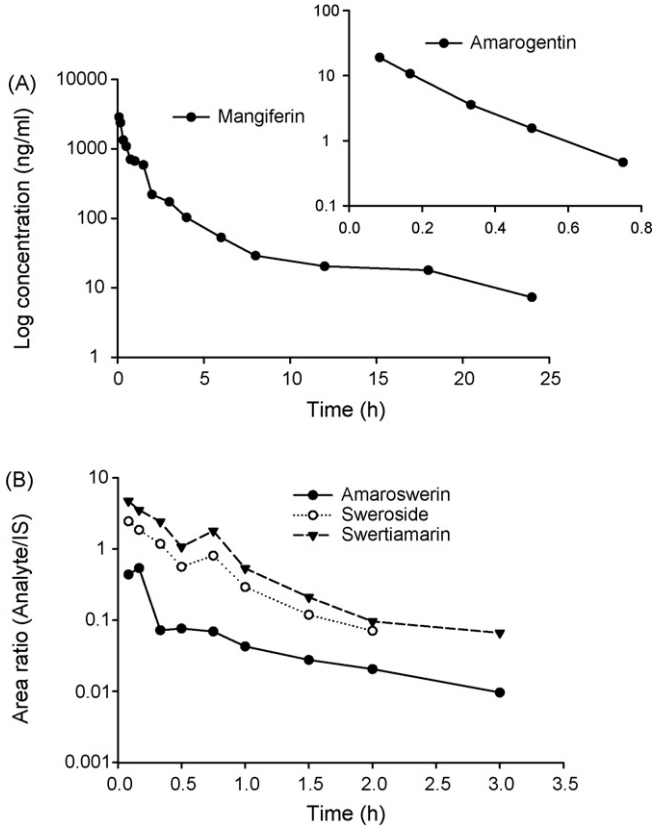

Fig. 4. (A) Plasma concentration time profile of marker mangiferin and amarogentin. (B) Area ratio–time curve of amaroswerin, sweroside and swertiamarin in rat plasma following i.v. (5 mg/kg) administration of herbal preparation CT ($n = 1$).

4. Conclusion

An LC–MS/MS bioanalytical method for simultaneous determination of two bioactive markers, mangiferin and amarogentin along with three other components, amaroswerin, sweroside and swertiamarin was developed and validated in rat plasma. The method was sensitive enough to detect low concentration of markers that is 1.56 ng/ml for mangiferin and 0.156 ng/ml for amarogentin in rat plasma. Kutkoside, a secoiridoid glycoside with similar structural nucleus was used as IS in assay method to account the variations due to matrix effect, extraction variability and instrument performance. Analytes recovery from spiked control samples were >90% except mangiferin where recovery was >60% using simple, convenient and fast sample clean up method. Intra- and inter-day accuracy and precision of the validated method were within the acceptable limits of <15% at low and <10% at other concentrations. The method was successfully applied to generate stability profile as well as PK evaluation of maximum number of components of chirata based herbal preparation in rat following i.v. administration.

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