Research Article

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RAPID LC-MS/MS METHOD FOR SIMULTANEOUS DETERMINATION OF COROSLIC ACID AND ASIATIC ACID FROM A MARKET FORMULATION OF LAGERSTROEMIA SPECIOSA LEAF EXTRACT SPIKED IN HUMAN PLASMA

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Abstract: Asiatic acid (AA) and Corosolic acid (CA) are two naturally occurring pentacyclic triterpenes that has shown numerous therapeutic activities and are active biomarkers of the commercially important plant *Lagerstroemia speciosa* (L). *Lagerstroemia speciosa* Linn., a Southeast Asian tree has shown wide range of therapeutic activities and is therefore used in numerous dietary supplements and herbal formulations. A research article has been published previously by the same authors for simultaneous quantitative determination of AA and CA from human plasma. The present research work involves an extension of this previous work wherein a sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for simultaneous analysis of AA and CA from a market formulation containing *L. speciosa* leaf extract spiked in human plasma. The analytes were extracted from the matrix using a simple solid-phase extraction procedure. Glycyrrhetinic acid (GA) was used as the internal standard for both analytes. A Kromasil C18 column provided chromatographic separation of analytes followed by detection with mass spectrometry. The method involves simple isocratic chromatography conditions and mass spectrometric detection in the negative ionization mode using a Waters Xevo TQS MS/MS system. The proposed method has been validated with a linear range of 1.00 – 1000ng/mL and 5 – 10000ng/mL for AA and CA respectively. The intra-day and inter-day precision and accuracy of the quality control samples were within the acceptance criteria of ±15% for both the triterpenoids. The total elution time was about 5 min which allows higher throughput. The assay values were found to be 0.88 % and 0.31% for Corosolic acid and Asiatic acid respectively in the market formulation containing *L. speciosa* leaf extract. This validated method can be applied for analysis of real samples from a bioequivalence study involving administration of formulations containing asiatic acid and corosolic acid as their active therapeutic components.

Keywords: Asiatic acid, Corosolic acid, LC-MS/MS, Solid phase extraction

INTRODUCTION

Lagerstroemia speciosa Linn. (Lythraceae), commonly known as Banaba or Tamhan, is a deciduous, tropical, flowering tree that grows widely in India, Philippines and South-east Asian countries. The tea from the leaves of *L. speciosa* has traditionally been used in the Philippines as a folk medicine for the prevention and treatment of diabetes [1,2]. In addition, *L. speciosa* extracts are also known to possess weight loss [3] and anti-oxidant effects [4,5] and is therefore used in numerous commercially available herbal formulations and dietary supplements. One such widely available dietary supplement containing *L. speciosa* leaf extract is marketed as "Banaba capsules" manufactured by Sushrut Ayurved Industries, Dharwad, and Karnataka, India. It is mainly used in the treatment of diabetes.

Pentacyclic triterpenes are the major and most important constituents of the herb *L. speciosa*. Different therapeutic effects of *L. speciosa* leaves are attributed to the active pentacyclic triterpenes, asiatic acid and corosolic acid [6]. AA has been reported to have many biological effects such as acetylcholinesterase inhibitory effect, enhancement of cognitive functions, protective activities against UV-induced photoaging, glutamate- or β-amyloid-induced neurotoxicity and hepatofibrosis[7-10]. CA exhibits a variety of pharmacological effects including anti-diabetic, anticancer, antiobesity, antiseptic and antioxidative stress properties [11-16]. In KK-Ay mice, an animal model of type-2 diabetes, CA could reduce the blood glucose levels and also significantly lower plasma insulin levels [17]. Additionally, clinical trials showed that CA could reduce the plasma glucose levels in human [18]. Therefore, to support preclinical and clinical development, determination of AA and CA from biological matrix such as human plasma is essential for evaluation of pharmacokinetic/pharmacodynamic parameters.

A sensitive, selective and reproducible HPLC/ESI-MS/MS method has been developed and validated by the same authors for simultaneous quantification of AA and CA using glycyrrhetinic acid (GA) as internal standard from the human plasma. The detection of

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both the components and IS was performed in same ionization (negative) mode without the use of any adduct ion formation. The present research work involves an extension of this previous work wherein a sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for simultaneous analysis of AA and CA from "Banaba capsules" extract spiked in human plasma.

EXPERIMENTAL

Chemicals:

HPLC grade methanol was procured from JT Baker, (USA). Reference standard of Asiatic acid (purity >95%) was purchased from Sigma-Aldrich Chemie (Aldrich Division; Steinheim, Germany). Corosolic acid reference standard (purity >95%) was purchased from Natural Remedies, Banglore, India. Ammonium acetate (GR grade), was procured from Merck Specialties Pvt. Ltd. (Worli, Mumbai, India).

Preparation of solutions:

The stock solutions of AA, CA and the internal standard GA were prepared in methanol at free base concentration of 1000µg/mL. Primary dilutions were prepared from stock solutions by dilution with water: methanol (40:60, v/v). The secondary dilutions and subsequent working solutions were prepared as and when required using the same diluent as those for the primary dilutions. These working standard solutions thus prepared were used to prepare the calibration curve and quality control samples.

A nine-point standard curve was prepared by spiking the previously screened blank plasma with appropriate amount of both AA and CA. The calibration curve ranged from 1.0 to 1000ng/mL for AA and 5.0 to 10000ng/mL for CA respectively.

Quality control samples were prepared at three concentration levels of 50, 400 and 850 and 100, 4000 and 8500 µg/mL for both AA and CA respectively in a manner similar to the preparation of calibration curve samples from the stock solutions.

Instruments and Equipments:

Solid phase extraction (SPE) was performed on an Ezypress 48 positive pressure SPE instrument (Orochem, Lombard, IL, USA). HPLC was performed on Waters Acquity Ultra performance LC system equipped with Acquity UPLC binary solvent pump and an auto sampler (Acquity UPLC Sample Manager – FTN). Chromatographic separation was performed on Kromasil 100-5C_{18} (150 x 4.6 mm, 5 µ) column with 5mM Ammonium acetate buffer: Methanol (25:75 v/v) as mobile phase. The flow rate was maintained at 0.6 mL/min and the auto sampler temperature at 10°C. The HPLC system was interfaced with Waters Xevo TQS triple quadrupole system equipped with an ESI source. MS detection was carried out in the negative ion mode. The already optimized ESI and MS parameters for detection of CA and AA published previously by the same authors were used for the present work. The data acquisition was done using Mass Lynx software version 4.1. Selective ion monitoring (SIM) mode was used for the quantification of the analytes monitoring precursor ion of m/z 487.42 for AA, m/z 471.3 for CA and m/z 469.26 for IS i.e. GA.

Sample extraction:

Dietary supplement: For analysis of the Banaba capsule, contents of 20 capsules were combined and 5gm was accurately weighed into a round bottom flask. 30 ml of methanol was added to the flask and the mixture was refluxed on a boiling water bath for about 30 min. The extract was then filtered through Whatman filter paper no. 41 (E. Merck, Mumbai, India). The same procedure was performed twice and filtrate obtained was combined together and made up to 100 ml with methanol. This solution was further diluted 1:1000 and then spiked in previously screened blank human plasma.

All frozen plasma samples (System suitability, blank plasma, zero standard, calibration curve standards and quality control samples) were thawed at room temperature and vortex mixed before analysis. Aliquots of 500µL of each plasma sample were transferred into eppendorf tubes followed by the addition of 25µL of IS dilution (5.000 µg/mL) in each tube except for double blank samples, to which 25µL of 50% methanol in water was added. To each tube was then added 500µL of 5 mM ammonium acetate buffer and vortexed for 30 seconds. Strata X cartridges (30 mg/mL Phenomenex, India) were conditioned with 1 mL methanol followed by 1 mL of HPLC Grade water. Plasma samples were then loaded onto the cartridges and allow them to elute out at constant flow of nitrogen stream at 2 psi. Washing was performed with 1 mL of 10 % methanol in water followed by 1 mL of HPLC water to clean up the loaded samples. Final elution was carried with 0.5 mL of mobile phase (5mM Ammonium acetate buffer: Methanol 25:75 v/v) and 10µL was injected into the LC/MS system.

RESULTS AND DISCUSSION

Selectivity:

Representative chromatogram obtained from blank plasma and plasma spiked with LLOQ (lower limit of quantification) standard for AA and CA respectively is presented in Figs.1 and 2, respectively. Similarly a representative chromatogram obtained from blank plasma and at its nominal concentration for GA (Internal Standard) is shown in Fig. 3. No interfering peak of endogenous compounds was observed at the retention time of analytes or the internal standard in

blank human plasma containing heparin as anti-coagulant from six different lots of normal controlled plasma and two each of lipemic and heamolysed plasma.

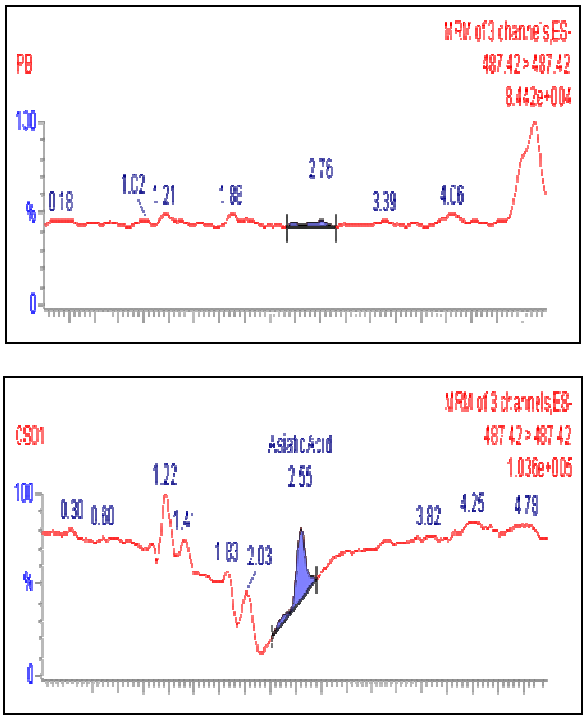

Fig.1: Representative chromatogram of plasma blank and plasma spiked with asiatic acid at the lower limit of quantification (1ng/mL).

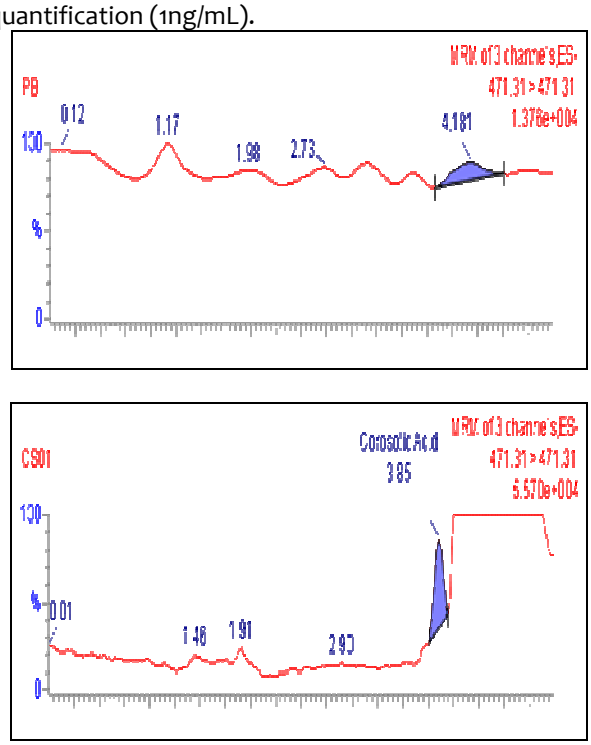

Fig.2: Representative chromatogram of plasma blank and plasma spiked with corosolic acid at the lower limit of quantification (5ng/mL)

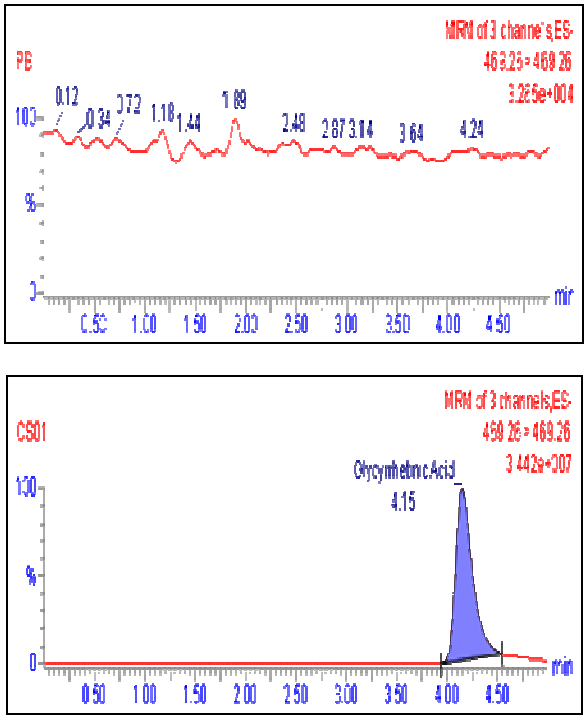

Fig.3: Representative chromatogram of plasma blank and plasma spiked with GA (about 50ng/mL).

Linearity:

The peak area ratios of calibration standards were proportional to the concentration of analytes in each assay over the nominal concentration range of 1.00 – 1000ng/mL and 5–10000ng/mL for AA and CA respectively. The calibration curves appeared linear and were well described by least squares lines. A weighing factor of 1/concentration^2 was chosen to achieve homogeneity of variance. The correlation coefficients were ≥0.995 for AA and ≥0.996 for CA. Across the nine points taken as calibration standards, the R.S.D. obtained over three batches was ≤3.8 and ≤3.0 for AA and CA, respectively.

Precision and accuracy:

The intrarun and interrun precision and accuracy results we found to be well within the acceptable limits as represented in Table 1 and Table 2.

Table.1: Intrarun precision and accuracy (n = 6) of AA and CA in human plasma

Analyte	Spiked conc. (ng/mL)	Mean calculated conc. with S.D. (ng/mL)	% R.S.D	% R.E.
AA	1.085	1.012 ± 0.0752	7.43	-6.76
	50.124	50.153 ± 3.2777	6.54	0.06
	401.238	396.001 ± 13.4493	3.40	-1.31
	850.874	823.462 ± 31.5012	3.83	-3.22
CA	5.121	5.280 ± 0.1762	3.34	3.10
	100.063	97.584 ± 7.3021	7.48	-2.48
	4051.042	4055.932 ± 143.2887	3.53	0.12
	8536.563	8320.715 ± 190.3740	2.29	-2.53

Table.2: Interrun precision and accuracy (n = 18) of AA and CA in human plasma

Analyte	Spiked conc. (ng/mL)	Mean calculated conc. with S.D. (ng/mL)	% R.S.D	% R.E.
AA	1.085	1.058 ± 0.0692	6.54	-2.49
	50.124	50.819 ± 3.4057	6.70	1.39
	401.238	396.623 ± 34.3077	8.65	-1.15
	850.874	815.935 ± 48.9137	5.99	-4.11
CA	5.121	5.117 ± 0.2128	4.16	-0.08
	100.063	101.038 ± 6.2317	6.17	0.97
	4051.042	4126.971 ± 269.4318	6.53	1.87
	8536.563	8416.852 ± 293.0076	3.48	-1.40

Recovery:

Six replicates at low, medium and high quality control concentration for the AA and CA were prepared for recovery determination. The mean recovery for AA and CA were 88.66% and 87.31% with R.S.D. values of 3.13% and 1.39%, respectively. The mean recovery for GA was 91.86% with an R.S.D. of 1.99%.

Use of the Validated Method for Quantification of Corosolic acid and Asiatic acid in dietary supplement containing *L. speciosa* leaf extract:

The plasma extract of "Banaba capsules" were injected into the LC-MS/MS system and the quantity of asiatic acid and corosolic acid were determined in terms of the percentage. The assay values were found to be 0.88 % and 0.31% for Corosolic acid and Asiatic acid respectively in the dietary supplement. The method is specific because it resolved both the standards well in the presence of other phytochemicals in the formulation. The method was found to be suitable for qualitative and simultaneous quantitative analysis of Corosolic acid and Asiatic acid from the formulation spiked in human plasma.

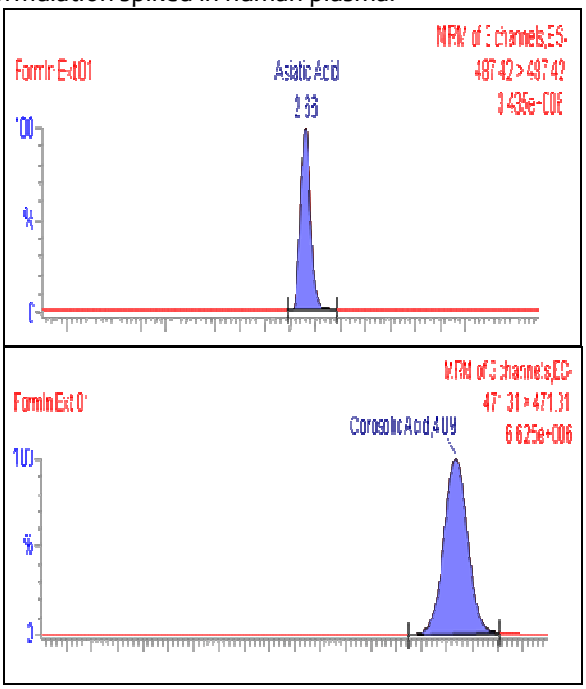

Fig.4: Representative chromatogram showing presence of asiatic acid and corosolic acid in human plasma plasma spiked with formulation

CONCLUSION

The method established in this work can be used as quality-control method for other market formulations or dietary supplements containing leaf extract of *L. speciosa*. Determination of pharmacokinetic and pharmacodynamic parameters for active components of plant extracts and herbal formulations is an important aspect for their wide acceptability in world market. This validated method can be applied for analysis of real samples from a bioequivalence study involving administration of formulations containing asiatic acid and corosolic acid as their active therapeutic components.

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