Simultaneous Estimation of Mangiferin and Amarogentin using Liquid Chromatographic Tandem Mass Spectrometry and its Application in Standardization of Herbal Preparation.

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Abstract

A sensitive and rapid liquid chromatographic/electrospray ionization tandem mass spectrometric assay method was developed and validated for the simultaneous quantification of mangiferin (MG) and amarogentin (AG) using dehydropregnenolone (IS) as an internal standard. The analytes were chromatographed on C$_{18}$ reversed-phase chromatographic column (5 $\mu$m, 30 x 4.6 mm i.d.) by isocratic elution. Mobile Phase consisted of acetonitrile: Milli-Q water with 0.5% glacial acetic acid at a flow rate of 1 ml/min. A short chromatographic run time of 3.5 minutes could be achieved with MG, AG and IS eluting at 0.3, 0.35 and 2.3 min respectively. The quantitation of the analytes was carried out using API 4000 LC/MS/MS system in the multiple reaction monitoring (MRM) mode. The calibration curve was linear over a dynamic range of 1.95-500 ng/ml for both the analytes. The method was validated in terms of linearity, sensitivity, specificity, accuracy and precision (Intra and Inter batch) for a period of five days. The limit of detection and lower limit of quantification were 0.98 and 1.95 ng/ml for both the analytes. The intra- and inter-batch accuracy and precision were found to be well within acceptable limits. The assay method was applied for the quality control analysis different batches of herbal preparation along with stability studies of MG and AG in simulated gastric acid fluid.

Introduction

Herbal medicines are still mainstay for primary health care because of better culture acceptability with the human body and lesser side effects. This widespread usage and interest is due to promising activity and efficacy shown by many herbal preparations in numerous clinical situations [1]. Currently existing approaches for quality assessment cannot fulfill the practical requirements of the safety and efficacy of herbal medicine [2-4]. In practice herbal preparation standardizations are based on one or more a number of biochemical constituents as either active or as marker compounds. Swertia chirata, a plant with bitter taste has been used in Ayurvedic system of medicines because of its febrifuge, anthelmintic, tonic and laxative properties and is prescribed for treatment of malarial fever [2]. Phytochemical investigations on the bioactive methanolic extract of defatted aerial part of S. chirata plant led to the isolation of the mangiferin (MG) along with secoiridoid glycosides i.e. amarogentin (AG).

MG, a xanthone-C-glycoside has a unique taxonomic character both in its distribution and biogenesis [3]. It has been reported to posses multiple biological activities like antitumor, antiviral, antioxidant, antidiabetic and immunomodulatory activity [4-6]. AG, a secoiridoid glycosides class of compound also possesses various biological activities such as chemopreventive, antibacterial, anticholinergic and antihepatitis activity and distribute in a restricted number of plant families served as important chemotaxonomic makers [7-10]. In
addition, owing to multiple biological activities and special taxonomic character of these particular compounds, prompted us to standardize methanolic extract of defatted whole plant of *S. chirata* (ME) in term of major components MG and AG.

The application of high performance liquid chromatography/ tandem mass spectrometry LC/MS/MS for this purpose, once considered too expensive, is now widely used and applicable, and generally gives methods with better selectivity and sensitivity [11,12]. LC/MS/MS allows separation and identification of individual molecules in complex samples often with reduced sample preparation and analysis time compared with other commonly employed techniques. In this context, electrospray ionization (ESI) is soft ionization method, and ESI-MS/MS has been shown to be a powerful method for identification of variety of plant constituents [13, 14].

There have been limited literature reports on the quantitative analytical method of MG and/or AG. One paper reported analysis of free MG in blood using microbore high-performance liquid chromatography and a limit of quantitation of 50 ng/ml for MG [15]. To best of our knowledge within limits of our literature survey, we report for the development and validation of a sensitive, selective, accurate and precise LC/MS/MS method using MRM in the negative ion mode for the simultaneous quantification of MG and AG. The method was applied to standardize various batches of ME with AG and MG as markers. Moreover, the stability of MG and AG in gastric fluid was also established by *in vitro* studies.

**Experimental**

**Chemicals and Materials**

Pure reference standard of MG and AG were obtained from the Division of Medicinal and Process Chemistry, CDRI, Lucknow, India. 16-dehydropregnenolone (IS) was procured from Pharmaceutics Division, CDRI. Sodium chloride (AR) was procured from s.d fine-chem limited, Mumbai, India. Potassium chloride (AR) was procured from Qualigens fine chemicals, Mumbai, India. Hydrochloric acid (AR) and glacial acetic acid (AR) were procured from E Merck (India) limited, Mumbai. Acetonitrile (HPLC grade) was purchased from Thomas Baker, Mumbai, India. Dimethyl sulphoxide (DMSO) was obtained from Ranbaxy Laboratories Ltd, Punjab, India. Ultra Pure water of 18.2 MΩcm was obtained from Milli-Q PLUS purification system (Millipore, USA).

**Plant material and herbal preparation**

*S. chirata* (Roxb ex flam) Karsh (Gentianaceae) grows abundantly at an altitude of 1220-3050 meter in sub himalayan region. The plant was collected in September-October by Botany Division, CDRI, Lucknow, India. Five batches of an herbal preparation ME in capsules form were provided by Clinical and Experimental Pharmacology Division, CDRI, Lucknow, India.

**Chromatographic Conditions**

A Perkin-Elmer Series 200 pump (Perkin-Elmer, USA) operated in isocratic mode was used to deliver a premixed mobile phase [solvent A: 90% acetonitrile in MilliQ water and solvent B: MilliQ water with 0.5% v/v glacial acetic acid in the ratio 60:40% v/v] at a flow rate of 1ml/min. The mobile phase was degassed for 20 min in an ultrasonic bath.
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(Bransonic Cleaning Equipment Company, USA) prior to the analysis. Chromatographic separations were achieved on Applied Biosystem® RP-18 column (30 mm x 4.6 mm i.d., 5 µm particle size). The samples (20 µL) were injected through a Perkin-Elmer series 200 auto injector on to the LC/MS/MS system with a 1:1 splitter. The analysis was carried out at ambient temperature and the pressure of the chromatographic system was 360-410 psi.

Mass spectrometric condition

The API-4000 (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada) mass spectrometer was operated using a standard ESI source coupled with a LC separation system. Analyst 1.3.2 was used for the control of equipment, acquisition and data analysis.

For optimization of MS parameters, approximately equimolar solutions (100 ng/ml) of each analyte were prepared in 50:50 ACN: water. Zero air (15 psi) was used a nebulizing gas and nitrogen as curtain gas (20 psi). The declustering potential (DP) was optimized for each analyte by constant infusion experiments using Harvard infusion pump, USA. Full-scan data acquisition was performed, scanning from m/z 400 to 650 in profile mode and using cycle time of 2s with step size of 0.1 unit in both positive and negative ion mode. The optimized DP’s for the respective analytes were set for the MS-MS experiments with nitrogen as the collision gas at a pressure of 3.0 x 10^{-3} millibars. Collision energies (CE) for fragmentation of precursor to product ions were optimized by constant infusion. The CEs for each of the analytes was optimized to obtain the most intense precursor to product ion transitions. MS-MS acquisition was performed by setting the mass of the analytes with appropriate scan range.

Utilizing this information, two or three intense product ions were selected to build an MRM method for quantitation and collision energies were optimized for different transitions by direct infusion.

Analytical standards and quality control samples

Individual standard stock solutions of MG (100 µg/ml) and AG (100 µg/ml) were prepared in ACN (0.2% v/v DMSO). Accurate amounts of AG and MG were weighed separately and dissolved in 200 µl of DMSO following which the volume was made up with ACN. Stock solution of IS (100 µg/ml) was prepared in ACN.

Mixed working stock (MWS) solutions of MG and AG, MWS (MG = 10 µg/ml and AG = 10 µg/ml) were prepared by transferring appropriate volumes of stock solutions to a 10 ml volumetric flask and making up the volume with ACN. MWS was used in the preparation of analytical standards. Working stock solutions of IS (20µg/mL) was prepared in ACN from the stock solution by appropriate dilution.

Analytical standards were prepared from MWS by diluting it with reconstitution solution [ACN: Milli-Q water, (50:50 v/v)] to obtain a concentration range of 500-1.95 ng/ml for both MG and AG. IS was spiked to each analytical standard to achieve a final concentration of 500 ng/ml.

Quality control (QC) samples at five different concentration levels, two low (QCL1, 1.95 ng/ml; QCL2, 3.9 ng/ml), two medium (QCM1, 15.6 ng/ml; QCM2, 62.5 ng/ml) and one high (QCH, 500 ng/ml) were prepared separately in five sets once each day and were used to assess accuracy, precision and linearity of the assay method.
Method Validation

Accuracy, precision, selectivity, sensitivity and linearity and were measured and used as the parameters to assess the assay performance [16].

Specificity

The specificity of the method was defined as non-interference in the regions of interest, for determination of the MG and AG in ME.

Limit of detection (LOD) and lowest limit of quantitation (LLOQ)

The LOD of assay method for different components quantity in sample corresponding to three times the baseline noise (S/N>3) and LLOQ was defined as the concentration with S/N>10.

Accuracy and precision

The accuracy of each sample preparation was determined by injection of analytical standard and five QC samples in pentaplet for five different days. The precision was determined by one-way ANOVA as within and between % RSD. The accuracy was expressed as %Bias.

Application: batch analysis for five different batches of ME

The method was applied to determine content of MG and AG five different batches (B-19, 27, 28, 29 and 42) of ME as a tool for standardization of these batches. A known concentration of ME (100 µg/ml) in Milli Q water with 10% acetonitrile was made for all the batches and the dilutions at low, medium and high concentrations were made in triplicate so that all the three lie within the analytical range. The concentration of MG and AG was directly read from the analytical standard curve % content (Mean ± SD) in test batches was determined.

Simulated Gastric fluid (SGF) stability study

NaCl (40 mM) and KCl (5 mM) were dissolved in MilliQ water and the pH was adjusted to 1.2 with hydrochloric acid. Human Dose of ME is 500 mg, which will produce a concentration of 2 mg/ml in stomach of normal healthy human being with 250 ml of gastric fluid. Batch-42 was employed in the stability studies. 20 ml SGF was preincubated in a shaking water bath for 10 minutes at 37 ± 2°C. 40 mg of ME (Batch-42) was added to 20 ml SGF so as to produce a concentration of 2 mg/ml for ME. Serial samples of 500 µl at 0, 5 min, 0.25, 0.5, 0.75, 1, 1.5 and 2 h were withdrawn from the incubation mixture under continuous shaking at 37 ± 2°C. Blank and control were also employed to check the degradation of MG and AG in absence of SGF. Test samples were suitably diluted and % remaining of MG and AG was determined using the validated LC/MS/MS method.
Results and Discussion

Optimization of LC conditions

Development of the method was initiated using RP-18 column (100 x 4.6 mm, id) and isocratic elution using a mobile phase containing acetonitrile: Milli Q water. For better resolution and sensitivity, varying % of ACN along, different gradient conditions, inclusion of ammonium acetate as aqueous phase were tried with no promising results. Furthermore, Cyano columns of different makes (Pierce and Phenomenex) were also tried to see the workability of the method but peak performance deteriorated for the analytes thus prompting the use of RP-18 columns for further consideration. Initially the elution was performed with 50% ACN in Milli Q water containing 0.5% glacial acetic acid at a flow rate of 1.0 ml/min which gave broad and late eluting peaks for IS (IS). There was not much effect on the retention times of MG and AG on increasing concentration of ACN but a considerable decrease in retention time and better peak shape and sensitivity for IS was observed. Among several modifications tried, the final LC conditions were optimized to ACN: Milli Q water (0.5% glacial acetic acid) (60:40 v/v) at flow rate 1.0 ml/min with MG, AG and IS eluting at 0.3, 0.35 and 2.3 min respectively.

Optimization of MS conditions

In order to find most sensitive ionization mode for compounds studied, positive and negative ESI were tested. MG (m/z, 422) and AG (m/z, 586) in positive ion mode gave sodium adduct at m/z 445 and m/z 609 respectively. Potassium adduct, though weak in intensity was observed for AG at m/z 625. Owing to division of signal between sodium, potassium and hydrogen ions, the sensitivity was compromised for both the analytes. Thus, infusion in negative ion mode was tried and strong signals of MG and AG were observed at 421 and 585 respectively. Thus, it was decided to use negative ion mode for the detection of these two components. DP were optimized to –85 V and –90 V for MG and AG respectively. MRM mode of detection, known for its high selectivity, was explored and MS-MS analyses were carried out to obtained product ion spectra of the analytes. The CE and CAD gas were optimized at 20 eV and 4 psi, respectively to get the most abundant ion in the product ion spectra. Wherever two prominent fragments were obtained, they were incorporated in MRM method to increase the sensitivity of the method and their summed ions were used for quantification [20]. MS condition for IS was optimized using direct infusion method. Utilizing this information, an MRM method for quantitation was developed and the, GS1, GS2 and temperature was optimized for different transitions by flow injection analysis (FIA).

Method Validation

Linearity and analytical standard range

The peak area ratios of MG and AG to the IS were found to be linear in their respective analytical range 1.95-500 ng/ml with %CV within 5% at all the concentration levels. The analytical model was selected based on the analysis of data by least square linear regression with and without intercepts (y = mx + c and y = mx) and weighting factors (1/x and 1/x²). The residuals improved by weighted (1/x²) least squares linear regression. The best fit for the analytical curve could be achieved by a linear equation of y = mx + c with a 1/x².
weighting factor. The correlation coefficient (R) for MG and AG were above 0.998 over the concentration range used.

Specificity

The method was found to specific for MG and AG with no interference in the elution zone of the analytes.

Accuracy and precision

The results show that the method is accurate, as %bias is within the acceptance limits. The precision around the mean value never exceeded 12% at any of the concentration studies.

Application

Batch Analysis

The validated method was employed for analysis of five different batches of ME (batch 19, 27, 28, 29 and 42. Batch 19, 28 and 42 were found to have 1.7±1.1 % of MG and 0.84±0.3 % of AG while Batches 27 and 29 were found to have much lower concentrations of these two markers. This variation is expected in herbal preparations and may be due to place and time of collection and processing.

Simulated Gastric Fluid (SGF) stability study

The validated method was employed for SGF stability study of MG and AG in ME. It was observed that both MG and AG were stable in acidic conditions for a period of two hours indicating their stability in SGF.

Conclusion

An LC/MS/MS method for the quantification of MG and AG was developed and fully validated over concentration range 1.95-500 ng/ml. This method has significant advantage in terms of sensitivity and selectivity, shorter run time (3.5min). The results of the assay performance and study conduct indicate that the method is precise and accurate enough for the routine quality control of various batches of ME. The method was also applied to establish the stability of MG and AG in SGF.

Reference

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