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Research Article

Simultaneous Determination Of Phyllanthin, Hypophyllanthin, And Niranthin In Rabbit Plasma By Lc-Ms/Ms

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ABSTRACT

Phyllanthus amarus Schum. et. Thonn. (Eurphorbiaceae) has hepatoprotective and anti-hepatitis B virus (HBV) activities. A simple, rapid, sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) method has been developed and fully validated to simultaneously quantify phyllanthin, hypophyllanthin, and niranthin in rabbit plasma using diazepam as an internal standard (IS). LC-MS/MS with an electrospray ionization (ESI) interface performed under the multiple reaction monitoring (MRM) mode was used to detect analytes. Simple liquid-liquid extraction was applied to extract analytes from rabbit plasma using n-hexane as an agent. The reconstituted samples were separated on a C18 column using the mobile phase composed of methanol-5 mM ammonium formate (63:37), which was isocratically delivered at a flow rate of 0.3 ml/min. A detailed validation of the method was performed in accordance with the guidelines of the US FDA and EMA. The calibration curves were established as linear in the range of 1–1,000 ng/ml for all analytes. The results of the intra- and inter-day precision and accuracy tests were well within the acceptable limits. Moreover, all analytes were stable under the test conditions. The validated bioanalytical method can be applied in bioavailability studies in rabbits after administration of Phyllanthus amarus.

Keywords: Hypophyllanthin, LC-MS/MS, Niranthin, Phyllanthin, Phyllanthus amarus.

INTRODUCTION

Recent years have seen a number of medical achievements in both treatment and prevention; however, infections have still been a leading cause of death, which affects the quality of life of millions of people worldwide, especially in developing countries, such as Vietnam. In particular, hepatitis B virus (HBV) - a viral infection - is an important issue. Nowadays, HBV treatment is mainly based on synthesis medicines (lamivudine, interferon, etc.), resulting in high cost and unwanted side effects [5], but herbal remedies with hepatoprotective activities have long been used, one being Phyllanthus amarus Schum. Et. Thonn. (Euphorbiaceae) - an herb that grows everywhere in Vietnam.

Phyllanthin (PLT), hypophyllanthin (HPL), and niranthin (NRT) are the main lignans in Phyllanthus amarus. Recent studies have shown that Phyllanthus amarus possesses anti-inflammatory, anti-oxidant, and anti-HBV properties, and it prevents liver toxicity due to alcohol and paracetamol overdose [4].

To the best of our knowledge, only one published work has simultaneously determined lignans in rabbits after administering Phyllanthus amarus using the liquid chromatography-tandem mass spectrometric (LC-MS/MS) method [3]. As an outcome, another new method that can simultaneously determine these lignans was necessary. Our purpose was to develop a quick, easy LC-MS/MS method to determine PLT, HPL, and NRT in rabbit plasma. This method can be used in future bioavailability studies on rabbits after administering Phyllanthus amarus.

EXPERIMENTAL SET-UP

Chemicals and reagents

The following reference standards were provided by the Institute of Drug Quality Control in Ho Chi Minh City: PLT (lot 020618, purity 99.3%), HPL (lot 010915, purity 97.92%), NRT (lot 010915, purity 97.02%), and diazepam (internal standard [IS], lot QT181 020213, purity 99.49%). LC-MS/MS-grade ammonium formate was purchased from Fisher Scientific (USA). The methanol and acetonitrile were LCMS-grade and obtained from J.T. Baker (USA).

Matrix

Blank rabbit plasma was provided by the Pharmacology Department at the Institute of Drug Quality Control in Ho Chi Minh City and was kept below -25 °C until used.

Preparation of standard solutions

Stock solutions of PLT, HPL, and NRT in methanol were used to prepare calibration-curve (CC) and quality-control (QC) samples from separate weighing and were stored below -20 °C. From these solutions, appropriate dilutions were made using methanol as a diluent to obtain working standard solutions at concentrations ranging from 20–20,000 ng/mL for all analytes.

The calibration standards and QC samples were prepared by diluting the previously mentioned working standard solutions 20 times with blank rabbit plasma. CC standards consisting of a set of 10 non-zero concentrations ranging from 1–1000 ng/mL were prepared. Similarly, the QC samples for each analyte were prepared at the following concentrations: 2, 40, 250, and 800 (low, middle-1, middle-2, and high QC, respectively) for all analytes. All the CC and QC samples were kept below -70 °C and brought to room temperature before used.

Internal standard preparation

The stock solution of diazepam (1.0 mg/mL) was prepared in methanol. On each day of analysis, an aliquot of the stock IS solution was diluted in methanol to obtain the IS working solution (1,000 ng/mL).

Samples and pre-treatment

Prior to use, the plasma samples were thawed and allowed to equilibrate at room temperature. An aliquot of 200 μL of rabbit plasma samples (blanks, CC, and QC samples) was spiked with a 10-μL aliquot of the IS solution (1,000 ng/mL of diazepam in methanol). After vortexing for 10 s, 2.5 mL of n-hexane was added, and the samples were vortexed for 1 min, shaken for 5 min, and then centrifuged for 5 min at 400 rpm. The clear organic layer was transferred to a glass tube and evaporated at 40 °C under a gentle stream of nitrogen. The residue was then reconstituted with 200 μL of methanol, vortexed for 1 min, centrifuged for 5 min (1860 × g; 0 °C), and filtered through a 0.22-μm membrane before an aliquot of 5 μL was injected into the LC-MS/MS system.

Analytical conditions

A triple quadrupole mass spectrometer LCMS-8040 coupled to a NEXERA X2 UHPLC (Shimadzu, Japan) was used in this study. The Shimadzu UHPLC system comprised an autosampler (SIL-30AC), two pumps (LC-30AD), a column oven (CTO-20A), and a controller (CBM 20A). Separation was carried out using a Gemini® 3 μm C18 column (dimensions: 150 × 3 mm) maintained at 40 °C. The mobile phase consisted of methanol and 5 mM of ammonium formate buffer (63:37, v/v) and was isocratically delivered at 0.3 mL/min.

Analysis was carried out in the positive ionization mode with an electrospray ionization (ESI) probe. The following parameters were optimal: an interface voltage of 4,000 V, a heatblock temperature of 400 °C, a desolvation line temperature of 250 °C, a nebulizer gas flow rate of 3 L/min, and a drying gas flow rate of 15 mL/min. Multiple reaction monitoring (MRM) experiments in the positive ionization mode were carried out to detect ion transitions. Quadrupoles Q1 and Q3 were set on unit resolution. The analysis data were obtained by LabSolutions software.

Table 1 shows the MRM transitions for each compound and other parameters.

Table 1: Optimal precursor and product ions and instrument parameters by compound

	Precursor ion m/z	Product ion m/z	Q1 (V)	Collision energy (V)	Q3 (V)
Phyllanthin (PLT)	436.00	151.10	-22	-33	-29
Hypophyllanthin (HPL)	261.20	231.10	-18	-14	-16
Niranthin (NRT)	449.95	369.20	-23	-18	-25
Diazepam (IS)	285.00	154.05	-16	-28	-27

Method validation

The method was completely validated in accordance with the US FDA [1] and EMA [2]

guidelines. The validation parameters determined were selectivity, linearity, precision, accuracy, matrix effect, recovery, dilution integrity, and various stability studies.

Selectivity

Six blank rabbit plasma sources were assessed to investigate interference peaks at the retention time of each analyte. The responses of the interfering peaks at the retention times of PLT, HPL, and NRT were acceptable if they were less than 20% of the response of the lower limit of qualification (LLOQ). The responses of the interfering peaks at the retention time of the IS were accepted if they were less than 5% of the response of the working IS.

Linearity and lower limit of quantification

Linearity was assessed using 10 calibration curves analyzed on separate days. For validation, the curves were constructed by calculating the peak area ratios of each compound to the IS and plotting these against the nominal concentration of the sample. The calibration curves were described by the linear equation $y=ax + b$, where y is the ratio of the analyte peaks and the corresponding IS peaks and x is the concentration (μg/mL); the regression coefficient, slope, and y-intercept of the resulting calibration curves were determined by weighted least squares ($1/x^2$) regression. Unknown concentrations were calculated from the equation of the calibration curve.

The upper limit of quantification (ULOQ) was defined as the highest standard concentration at which both the relative standard deviation and the percent deviation from the nominal concentration were less than 15%. The LLOQ was defined as the lowest concentration that could be measured with an inter-day coefficient of variation (CV) within ±20% and an accuracy between 80-120%.

Matrix effects

Six sources of blank rabbit plasma were treated and analyzed as separate samples in six replicates at high and low QC concentrations. The matrix effects for each analyte and the IS were determined separately in each sample by determining the peak area in the post-extraction spiked plasma to the peak area in the spiked methanol. The method's specificity was also tested by screening the six drug-free rabbit blank plasma lots.

Accuracy and precision

The accuracy and precision of the analytical method were determined by analyzing samples at the LLOQ, low quality control (LQC), medium quality control-1 (MQC-1), MQC-2 and high quality control (HQC) concentrations and prepared as described above. Six replicates of each sample level were quantified in one run for the intra-day accuracy and precision. Six replicates of each sample level were assayed within three days (a total of 18 replicates for each concentration level).

Recovery

The recovery (extraction efficiency) of the analytes was determined by analyzing spiked QC samples at high, middle, and low concentrations. Six replicates of each QC sample were prepared by the aforementioned procedure and injected into the LC-MS/MS system. The recovery was determined by comparing the peak area resulting from the extracted spiked plasma samples with the peak area resulting from the samples spiked in methanol at the same concentration levels.

Dilution integrity

The accuracy and precision of the diluted samples were determined by performing 2-fold dilutions of a highly concentrated solution (1600 ng/mL for all analytes) with drug-free rabbit plasma. The calculated concentrations were compared to the nominal concentrations.

Carryover

Carryover was assessed by injecting blank plasma immediately following the injection of the ULOQ. Carryover was accepted if it was less than 20% of the LLOQ of each analyte and less than 5% of the IS response for an IS.

Stability

The short-term, long-term, freeze-thaw, and post-preparative stabilities of PLT, HPL, and NRT in plasma were examined using low and high QC samples and calculating the bias between observed and theoretical concentrations.

The short-term stability was investigated by analyzing six replicates of the samples at each concentration (-70 °C) at room temperature for 6 h before sample preparation. The long-term stability was evaluated by keeping six replicates of the low and high QC samples at each level with storage at -70 °C for 30 days. The freeze-thaw stability was determined over three cycles. In each cycle, the spiked plasma samples were frozen at -70 °C for 24 h and thawed at room temperature. The post-preparative stability was examined by keeping six replicates of each low and high concentration levels in the autosampler at 5 °C for 24 h. The samples were analyzed, and the results were compared with nominal concentrations. The

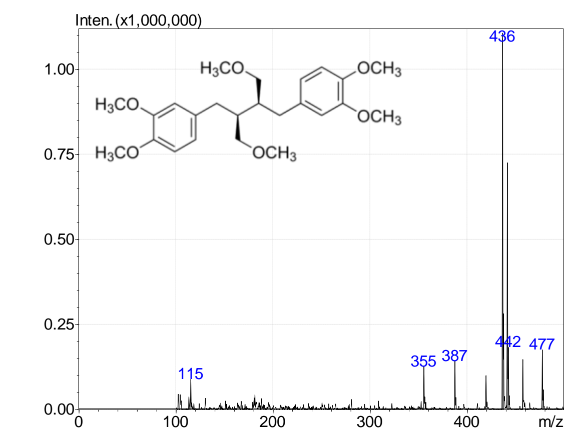
stabilities were acceptable if the biases (%) were within ±15 %.

RESULTS AND DISCUSSION

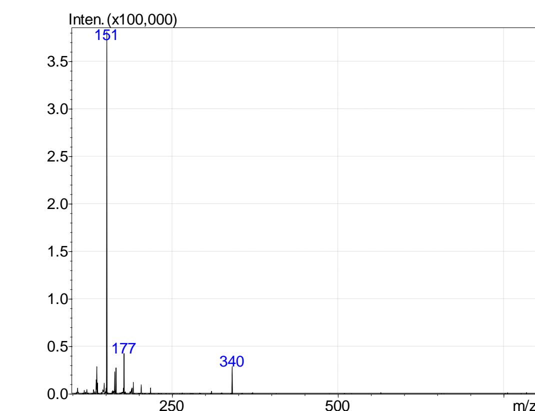
Method development

During the method development, ESI was operated in the positive ionization mode. The Q1 MS full-scan spectra for PLT, HPL, NRT, and diazepam (the IS) predominantly contained protonated precursor $[M+H]^+$ ions at m/z 436.00, 261.20, 449.95, and 285.00, respectively.

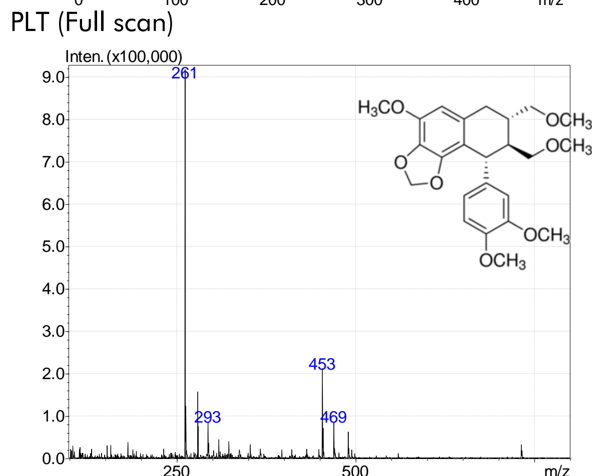
The most abundant product ions in the Q3 MS spectra for the PLT, HPL, NRT, and IS were observed at 151.10, 231.10, 369.20, and 154.05, respectively. Figure 1 shows the Q3 MS spectra m/z of the analytes and IS. The MS/MS parameters were systematically optimized for each analyte and the IS to obtain a consistent, adequate response. A dwell time of 100 ms was sufficient.



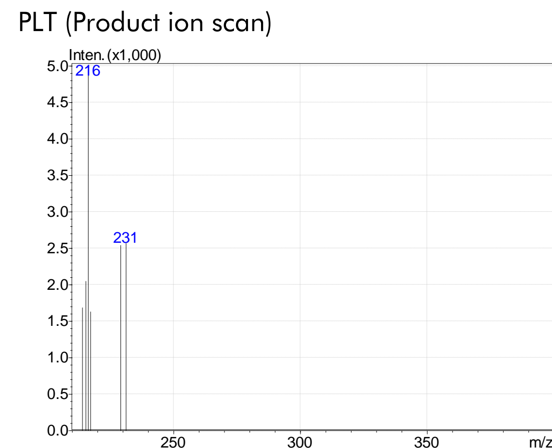
PLT (Full scan)



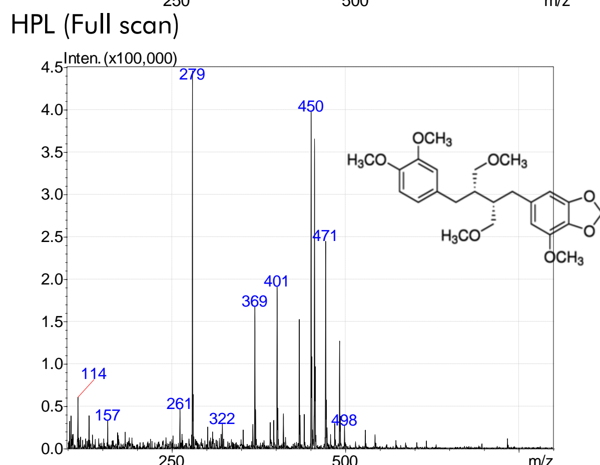
PLT (Product ion scan)



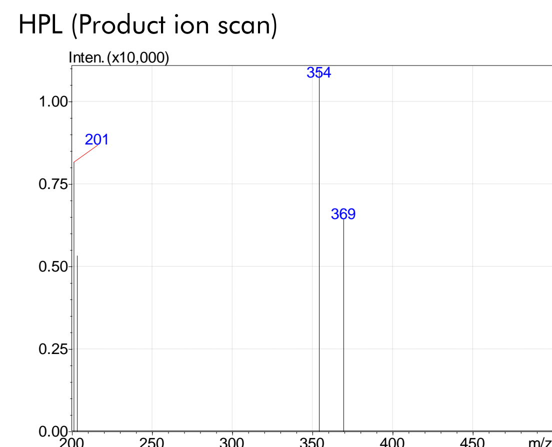
HPL (Full scan)



HPL (Product ion scan)



NRT (Full scan)



NRT (Product ion scan)

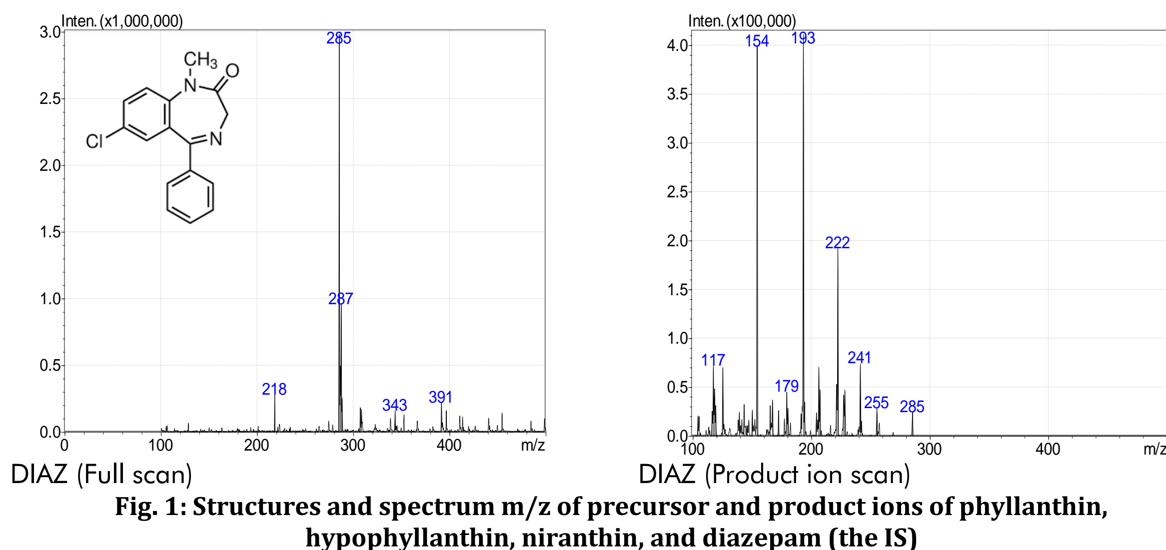


Fig. 1: Structures and spectrum m/z of precursor and product ions of phyllanthin, hypophyllanthin, niranthin, and diazepam (the IS)

The chromatographic conditions were investigated by considering the column type, mobile phase component (methanol/acetonitrile along with ammonium formate/ammonium acetate buffer or a solution of formic acid with concentrations of 0.05%, 0.1%, and 0.2%), the pH (3-7) and strengths (2-8 mM) of the buffer, and flow rate (0.2-0.5 mL/min). Thus, we changed these parameters to obtain a symmetrical peak shape, short run time, minimum matrix interference, and solvent consumption. The investigations revealed that a mobile phase composed of acetonitrile resulted in a poor peak shape, and significant decreases in the peak responses for all the analytes and IS were achieved with a buffer containing ammonium acetate or formic acid.

Based on the outcome of various trials, the Gemini® C18 (100 × 2 mm; 3 μm) column was selected. The mobile phase consisting of acetonitrile-5 mM ammonium formate buffer (63:37, v/v) at a flow rate of 0.3 mL/min ensured a symmetrical peak shape and the separation of all the analytes and IS. Figures 2 and 3 show the chromatograms of the blank plasma and analytes at the MQC level with the IS. The blank plasma chromatogram was obviously clean, with no endogenous interfering peaks at the retention times of the analytes or IS.

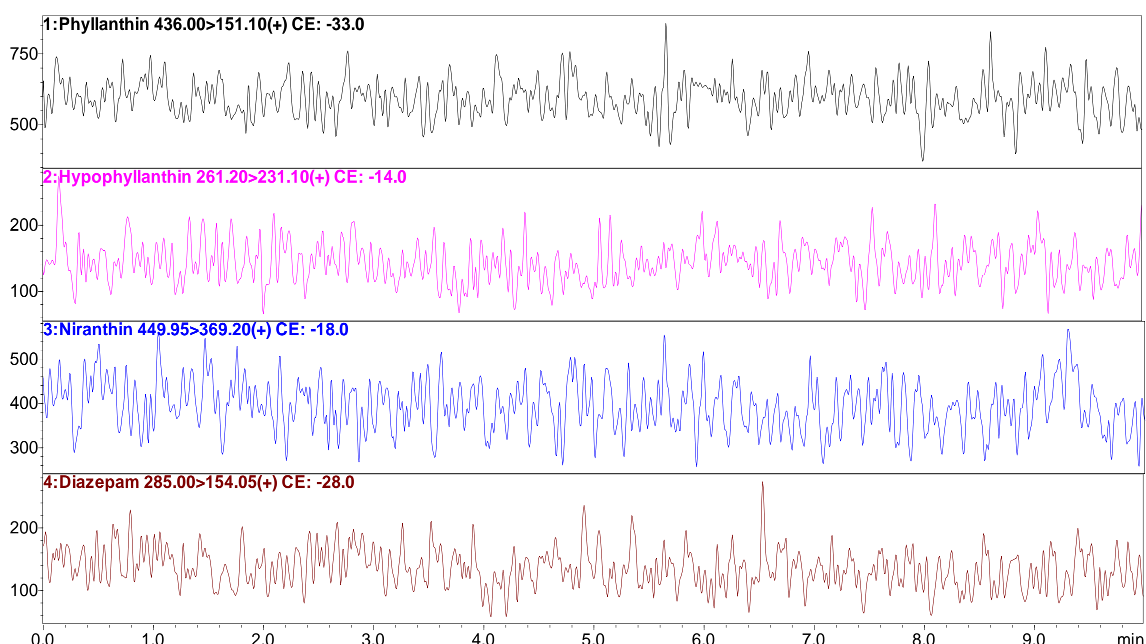


Fig. 2: Chromatogram of blank rabbit plasma

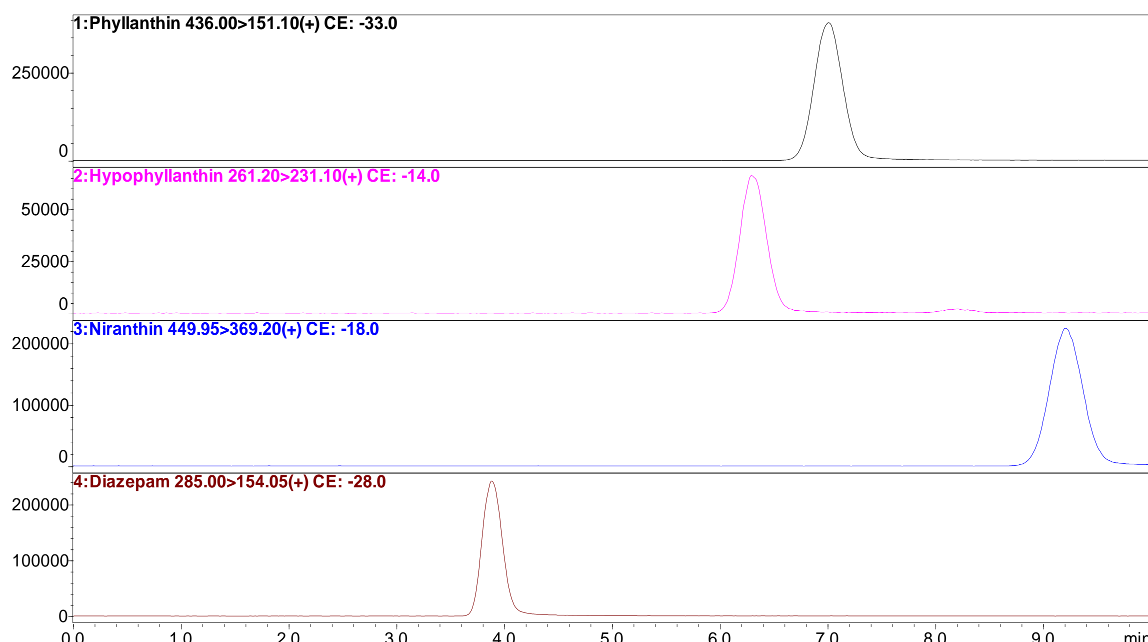


Fig. 3: Chromatogram of plasma sample at concentration of MQC (500 ng/mL for all analytes) and the IS

Extraction procedure

Protein precipitation (PP) and liquid-liquid extraction (LLE) are routine sample pre-treatment strategies. During the initial stages of method development, the PP method was carried out using methanol and acetonitrile as agents, but it failed to achieve a clean extract and produced more background noise with poor sensitivity. Therefore, LLE was desired not only to purify but also to concentrate the sample.

LLE was initiated with methyl tert-butyl ether (MTBE), ethyl acetate, and n-hexane. The agent n-hexane showed more significant recoveries (>90% for all analytes) than MTBE (approximately 60%) or ethyl acetate (approximately 50%). Therefore, n-hexane was finally chosen as the agent for LLE

The volume of n-hexane was also evaluated from 2-3 mL. A volume of 2 mL showed lower recoveries for all the analytes and IS than a volume of 2.5 mL and 3 mL. However, 3 mL of n-hexane created ion suppression for all the analytes, and the matrix effect was not within the acceptable limits per the guidelines.

Based on the trials, LLE with 2.5 mL of n-hexane was chosen to extract PLT, HPL, NRT, and the IS from the rabbit plasma samples.

Method validation

The method was successfully validated per the US FDA and EMA guidelines. Table 2 summarizes the results of the method validation.

Table 2: Summary of method validation results

No.	Parameters	Results
1	Selectivity/ Specificity	The LLOQ samples were chromatographed, and the retention times of PLT, HPL, NRT, and the IS were 6.95, 6.27, 9.20, and 3.89 min, respectively. No significant interference in blank plasma traces was observed at the retention time of the analytes and IS.
2	Lower limit of quantification	The lowest measurable concentration with acceptable precision and accuracy for all analytes was 1 ng/mL for all the analytes, which was set as the LLOQ for the analytes. At this concentration, the signal-to-noise ratio (S/N) was found to be >10 for all the analytes.
3	Linearity	The analytes showed good linearity in the concentration range of 1-1,000 ng/mL with the coefficient of determination (R^2) >0.99 for all the analytes. The accuracy of the back-calculated concentrations for the calibration standards ranged from 97.55–105.16%, revealing good linearity. The equations are as follows: PLT (1-1,000 ng/mL): $y = 0.0495x + 0.0053$; $R^2=0.9977$ HPL (1-1,000 ng/mL): $y = 0.0086x - 0.0008$; $R^2=0.9990$

		NRT (1–1,000 ng/mL): $y = 0.0333_x - 0.0065$; $R^2=0.9965$
4	Intra-day and inter-day precision and accuracy	The intra-day (n=6) and inter-day (n=18) accuracy and precision are the following: PLT (91.49-110.79%; CV: 2.17-5.71%) HPL (100.30-110.01%; CV: 2.69-3.28%) -NRT (88.78-111.07%; CV: 2.17-3.75%) The results showed good precision and accuracy.
5	Recovery	The recovery was evaluated at three levels (low, middle-2, and high QC). The results are shown below: PLT (89.48-100.69%; CV: 2.05-4.53%) HPL (88.42-102.04%; CV: 1.56-7.35%) NRT (99.36-103.73%; CV: 1.22-4.55%) IS (93.79-98.07%; CV: 2.06-5.20%) Good recoveries were obtained for the analytes and IS. The CV ranged from 1.22% to 7.35%, which showed consistent extraction recoveries.
6	Stability	The stock solutions were found to be stable for at least 30 days at below -20 °C. Also, a wide range stability experiments, namely, benchtop stability (room temperature for 6 h), freeze-thaw cycles (3 cycles), an autosampler (5 °C for 24 h) and long-term stability at -70 °C for 30 days. The mean percent nominal values were found to be within ±15% of the measured concentrations for the analytes at their LQC and HQC levels, and the precision values were within ±15%.
7	Matrix effect	The results were well within the acceptable limits, as the IS-normalized matrix factors of PLT, HPL, and NRT at the concentrations of LQC and HQC were 3.57-7.64%, 3.07-12.18%, and 3.32-8.57%, respectively.
8	Carryover	No carryover was found in the blank plasma sample after injecting the ULOQ sample, which indicated that the proposed assay was not compromised due to carryover.
9	Dilution integrity	The accuracy values of PLT, HPL, and NRT were 108.03%, 106.94%, and 108.62%, respectively, and their precision values were 2.28%, 3.46%, and 2.90%, respectively. Therefore, the samples with concentrations above the ULOQ (1000 ng/mL) can be quantified by performing a half dilution (1:2) with drug-free blank rabbit plasma.

CONCLUSION

The LC-MS/MS assay reported here is simple, rapid, specific, and sensitive for simultaneous quantifying PLT, HPL, and NRT in rabbit plasma, and it is fully validated according to US FDA and EMA guidelines. The simple extraction involving n-hexane as an agent gave high, consistent, reproducible recoveries for the analytes from rabbit plasma. The method also provided good linearity. The stability of the analytes in plasma and
in methanol under different conditions has been extensively evaluated.

From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability studies of products or herbal medicine using Phyllanthus amarus with the desired precision and accuracy.

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