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Rapid RP-HPLC technique for the determination of phyllanthin as bulk and its quantification in *Phyllanthus amarus* extract

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Abstract

A simple, specific and precise high performance liquid chromatographic method has been developed and validated for the estimation of Phyllanthin, an important bitter lignan present in *Phyllanthus amarus*. Furthermore, the developed method was used to successfully quantify the Phyllanthin in the plant extract also. The mobile phase optimized for the RP-HPLC was ethanol-water 66:34 (%v/v) and that was very simple and cost effective. The detection was carried out using variable wavelength UV–VIS detector set at 229 nm. Linearity for the developed method was found over the concentration range 1–50 µg/ml with a correlation coefficient of 0.999. Method was validated according to ICH guidelines and found to be suitable for rapid quantification of Phyllanthin in bulk and in plant extracts.

Keywords: *Phyllanthus amarus*, Phyllanthin, HPLC and Plant extract.

Introduction

Herbal drugs due to economical concern, considered as safe and easy in availability have been extensively used traditionally in folk medicine worldwide and particularly in under developed area like African continent and developing countries in Asian region like India and china. Furthermore, herbal drug's wealth in these regions is extremely good. Biologically active compounds from herbal sources have always been a great interest for scientists working on infectious and non-infectious diseases. *Phyllanthus amarus* commonly known as Jangli amli (in Hindi) is a medicinally important plant that belongs to the family Euphorbiaceae. This plant is used ethnomedicinally as antiviral, antihyperglycemic, antioxidant and hepatoprotective [1-15]. These activities of *Phyllanthus amarus* are mainly a result of the presence of lignans and polyphenols, especially flavonols [16]. Beside individual parts of this plant like roots, leaves, fruits, milky juice, the whole plant is used in medicinal preparations. Phyllantin, a bitter lignan is a major constituent it is obtained from root and the bark part of the *Phyllanthus amarus* [17]. A validated sensitive quantitative method is a precondition for phytoconstituents of appropriate quality as well as for pharmacological activities of standardized drug preparations. Now a days HPLC is often used for the quantification of phytoconstituents because of its sensitiveness i.e. very low concentration of the drug can easily be detected, so can be applied in *in-vitro* and *in-vivo* studies. So, this paper describes the development and validation of RP-HPLC for assay of Phyllantin in bulk and further quantifies the Phyllantin in *Phyllanthus amarus* collected from our university (Hamdard University, New Delhi-62) campus. Present paper describes a RP-HPLC method, which is simple, specific, precise and rapid with

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very short retention time. So it can be applied in routine analysis of the drug.

Material and Methods

Chemicals and reagents

Phyllanthin was gifted from SPIC Pharma, Chennai, India. For the extraction purpose, plant of *Phyllanthus amarus* was collected from herbal garden of Jamia Hamdard, New Delhi-62, India, in the month of July. Ethanol (HPLC grade) was purchased from Merck, Mumbai, India. The HPLC grade water was prepared by Millipore mili-Q (Bedford, MA, U.S.A.). Other chemicals and reagents were of AR grade. All solutions used for the analysis were filtered through 0.45 µm membrane filter using Millipore filtration unit.

Apparatus

Chromatographic measurements were made on Breez Liquid chromatographic system (Tokyo, Japan) which consisted of a solvent delivery pump (model LC-10AD), injector (Model SC), UV–visible absorbance detector (model SPD-10A) and the instrument was connected to the computer with Class-VP 5.032 software.

Chromatographic conditions

HPLC analysis was performed on a lichrosorb C18 analytical column (250mm X 4.6mm i.d., 10µm; Shiseido Fine chemicals, Japan). The mobile phase was consisted of ethanol and water in different ratios and pumped at a flow rate of 1.0 ml/min. The detection was carried out at 229 nm. An injection volume of the sample was 20µl. The temperature in laboratory was at 25 ± 2°C.

Standard Solution

Standard stock solution of Phyllanthin (100µg/ml) was prepared in ethanol. The prepared solution was stored at 4°C protected from light. Working standard solutions were freshly obtained by diluting the stock standard solutions with mobile phase during the time of analysis.

Calibration standards and quality control samples

Calibration standards were prepared at concentrations of 1, 2, 4, 8, 10, 20 and 50µg/ml from a standard solution of 100µg/ml by appropriate dilution with mobile phase. Four quality control (QC) samples at the concentrations of 1, 1.5, 25 and 40µg/ml representing the LOQ, low, medium and high concentrations, respectively, of the linearity range were prepared from the standard solution.

Preparation of the ethanolic extract of *Phyllanthus amarus*

1.0g air dried aerial parts of *Phyllanthus amarus* was extracted in a soxlet for 12 hours separately at room temperature (25 ± 5ºC) with 10 ml of ethanol. After filtering, the filtrate was taken in a conical flask, while residue was again extracted with ethanol. The same process was repeated twice. All the filtered ethanolic extracts were combined and concentrated under reduced pressure and low temperature of 40°C on rotary evaporator. Filtered through 0.45 µm membrane and made up to 1ml with Mobile phase prior to RP-HPLC analysis.

Validation

The developed RP-HPLC method was validated by determination of selectivity, linearity, limit of quantitation and detection, precision, accuracy, recovery, robustness and stability as per the ICH guideline [18].

Results and Discussion

A variety of mobile phase in different ratios were investigated in the development of an RP-HPLC method for the analysis of Phyllanthin. These included ethanol-water, 90:10 (%v/v), ethanol-acetonitrile, 70:30 (%v/v), acetonitrile-water, 70:30 (%v/v), ethanol-water, 50:50 (%v/v), ethanol-water, 80:20 (%v/v), ethanol-phosphate buffer, 25:75 (%v/v) pH 4.5-6.5 and acetonitrile-phosphate buffer, 25:75 (%v/v) pH 4.5-6.5. The final selection of mobile phase was decided on the basis of sensitivity of the assay, retention time of the drug, solubility of drug, ease of preparation, availability and cost effectiveness of the solvents. Phyllanthin is poorly water soluble

drug and is almost insoluble in aqueous solutions, whereas it is soluble in organic solvent like methanol. The RP-HPLC method was optimized with a view to develop a method for assay of drug in bulk and formulation. The method is also applicable to assay the Phyllanthin. Among several mobile phase investigated, ethanol-water 66:34 (%v/v) was found to produce sharp, well defined peak with very good symmetry(1.04) and low retention time (6.925minute). with the change of flow rate from 1.5ml/min to 0.5ml/min, retention time was around 12min which is higher than the Rt 6.925 (at 1.5ml/min). When flow rate changes to 2 ml/min, retention time obtained was 7min. In both the cases the consumption of mobile phase is higher than the amount consumed at a flow rate of 1.5ml/min. Hence, the mobile phase was optimized as ethanol-water 66:34 (%v/v) at a flow rate of 1.5ml/min with the retention time of drug around 6.925 min and theoretical plate 20685 which shows the good sensitivity of the developed method (Figure. 1). The UV spectrum of Phyllanthin shows the highest absorbance at 229nm in solution made up of mobile phase (data were not given). So, Quantification was achieved with UV detection at 229nm based on the peak area. Chromatograms obtained for the Phyllanthin standard and extract revealed that they had similar pattern as shown in Figure 1 and 2. Linearity was evaluated by determining working concentrations containing 1, 2, 4, 8, 10, 20 and 50µg/ml of Phyllanthin (Figure. 3). Peak area and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. The regression equation was y=22899 x (where y is the response and x the amount of Phyllanthin). Linearity was found over the concentration range 1–50 µg/ml with a correlation coefficient of 0.999. The linearity of the calibration curve was validated by a high value correlation coefficient. DL (Limit of detection) and QL (quantification limit) of the method were found to be 19.97 ng/ ml and 60.596 ng/ml, respectively, which indicate that the proposed method can be used for detection and quantification of Phyllanthin in a very wide concentration range. The recovery rate was 98.79% as calculated by addition of known amounts of standard Phyllanthin to the plant extract.

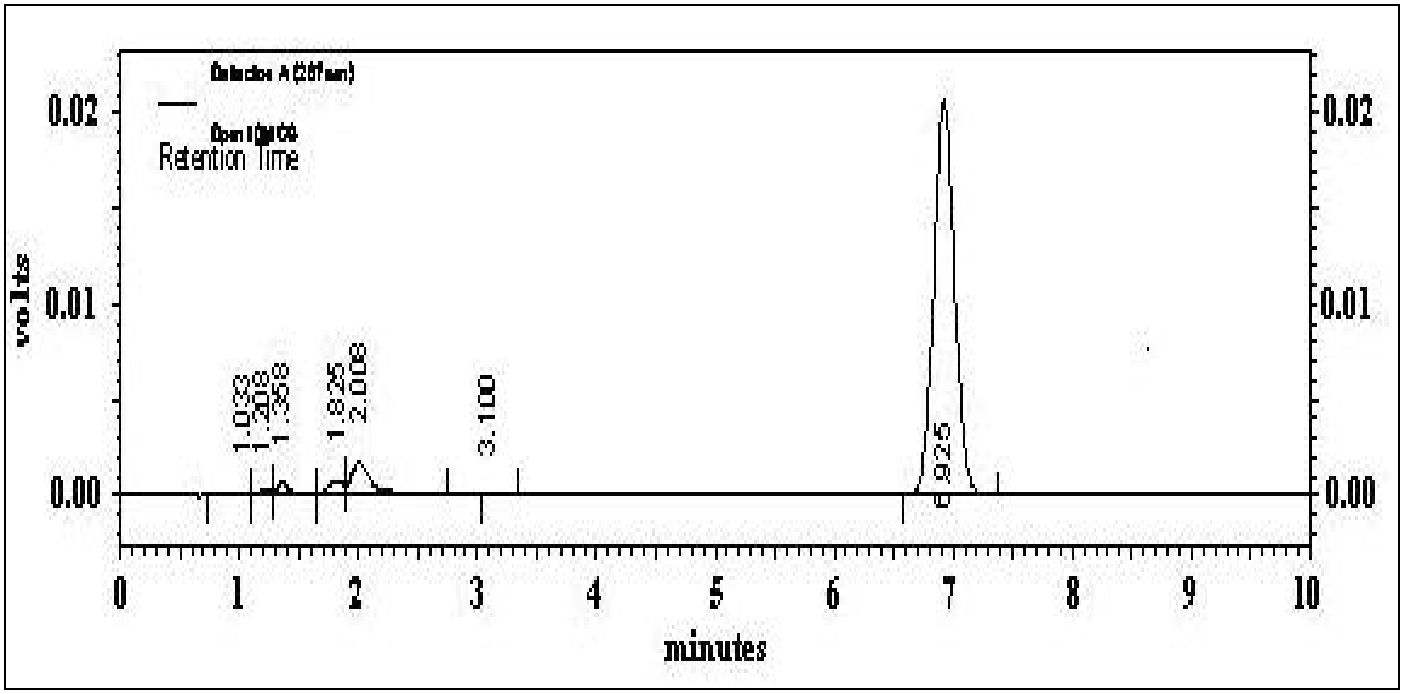

Figure 1: Chromatogram of Phyllanthin in the mobile phase; ethanol and water in the ratio of 66:34 (%v/v).

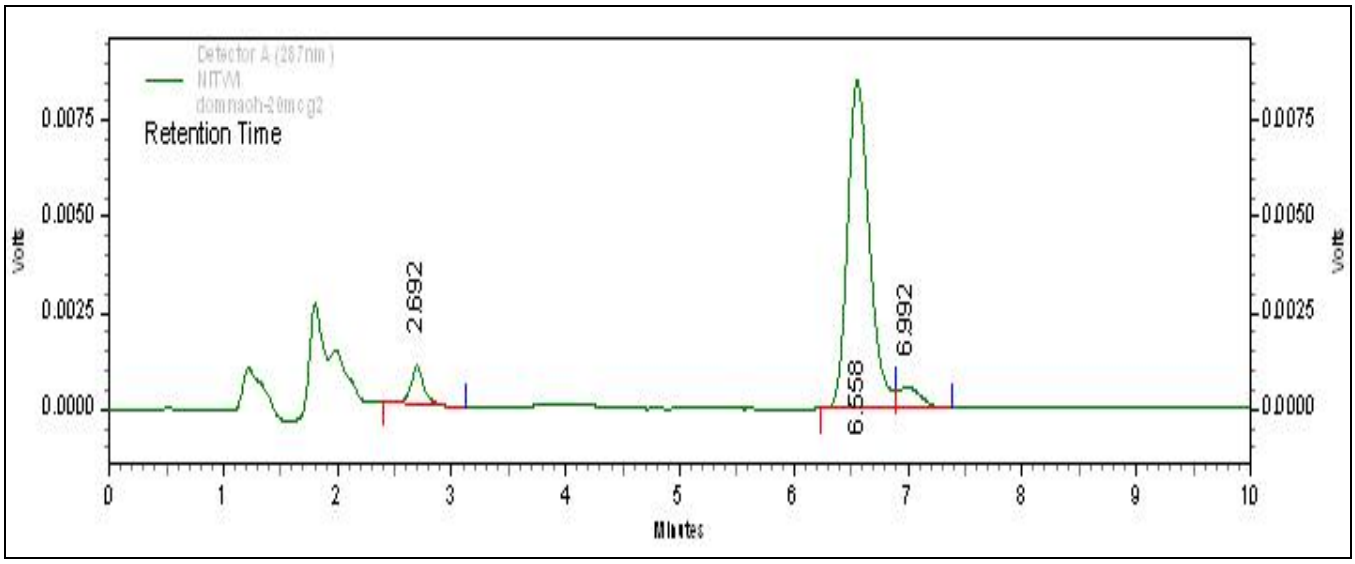

Figure 2: Chromatogram of *Phyllanthus amarus* extract in the mobile phase; ethanol and water in the ratio of 66:34 (%v/v)

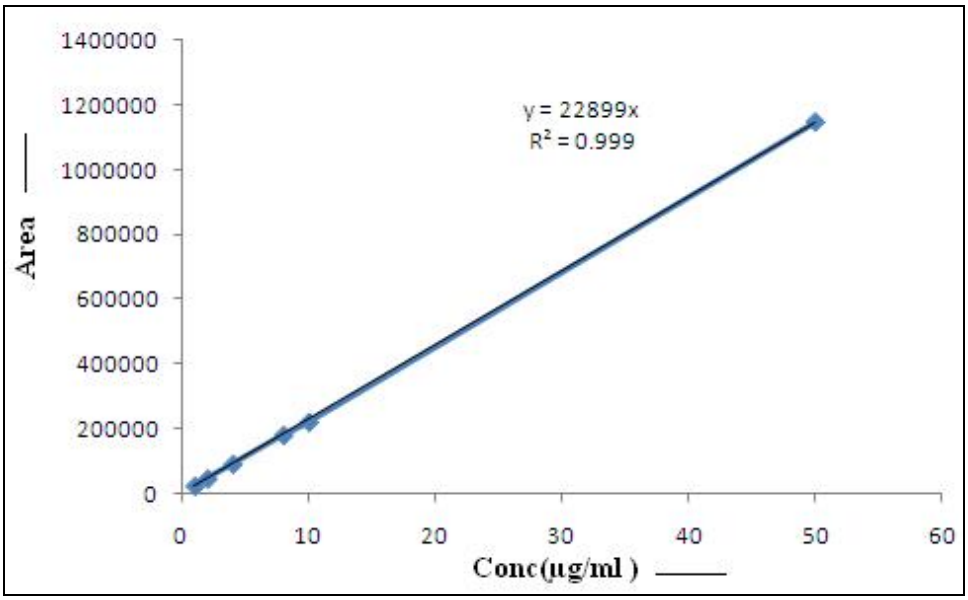

Figure 3: Calibration curve of Phyllanthin in in the mobile phase; ethanol and water in the ratio of 66:34 (%v/v) by reverse phase high performance liquid chromatography (RP-HPLC) method.

Conclusion

Proposed HPLC method is rapid, direct, specific, accurate and precise for determination of Phyllanthin in bulk and in *Phyllanthus amarus* extract. Because of short chromatographic run time (10min), the developed method can be adopted for the routine quantification of Phyllanthin herbal extracts and in *in-vivo* animal studies.

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