

Pharmacokinetic study of phyllanthin and hypophyllanthin after oral administration to rats

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ABSTRACT

Objective: The present study was carried out to develop a sensitive and cost effective HPLC method for the determination of bioactive lignans (phyllanthin and hypophyllanthin) and its application in a pharmacokinetic study. **Methods:** Identification of lignan compounds on C-18 column was monitored at a range of 199–400nm using photodiode array detector (PDA) with methanol-water (66:34, v/v) as mobile phase at a flow rate of 1ml/min. Carbamazepine was used as internal standard. **Results:** From the developed method LOD and LOQ values were found to be 56.14ng/ml and 169.99ng/ml for phyllanthin, and 56.04ng/ml and 169.82ng/ml for hypophyllanthin. The validated RP-HPLC method herein was applied for pharmacokinetic studies and C_{max} (ng/ml) values for administered three oral doses (2.5, 5 and 10mg/kg) of phyllanthin and hypophyllanthin were 0.28 ± 0.06 , 0.53 ± 0.16 , 0.98 ± 0.22 and 0.68 ± 0.76 , 1.35 ± 0.23 , 2.45 ± 0.33 , respectively. **Conclusion:** In conclusion, developed HPLC-PDA method effectively determined the phyllanthin and hypophyllanthin in various solvent and plasma samples. This method was successfully applied in conducting their oral pharmacokinetic studies.

Keywords: *Phyllanthus amarus*, phyllanthin, hypophyllanthin, HPLC-PDA, pharmacokinetics.

INTRODUCTION

Phyllanthus amarus Schum and Thonn (Euphorbiaceae) is widely distributed in tropical areas of world such as China, Java, Southern Florida, Bahamas, West Indies and Tropical America.^[1] *Phyllanthus* genus is having significant ethnomedical importance^[2,3] and is officially listed in Indian herbal medicine system of Ayurveda. *P.amarus* was used for the treatment of inflammation,^[4] liver ailments,^[5] free radical scavenging activity,^[6] kidney stones^[7] and also acts as immunomodulatory agent,^[8] anticancer agent,^[9] antiviral agent^[10] and HIV replication and reverse transcriptase inhibitor agent.^[11] *P.amarus* is well known for its biologically active compounds. The main active constituents of the herb are lignans, terpenes, sterols, flavonoids, alkaloids,

volatile oils and tannins.^[12] Lignans like phyllanthin and hypophyllanthin (Fig. 1) from *P.amarus* found to have potent bioactive nature in the treatment of several diseases like cancer,^[13] hepatic disorders,^[14] free radical scavenging activity^[15] and inflammation.^[16]

Pharmacokinetic studies are essential for fixing the dose and for studying the bioavailability. However, till now, research on phyllanthin and hypophyllanthin was focused on pharmacological effects while their pharmacokinetic studies were unclear. Therefore, the aim of the present paper was to investigate the pharmacokinetic properties of phyllanthin and hypophyllanthin at different doses.

High performance liquid chromatography (HPLC) is a powerful tool for natural product chemists in the separation and identification of very close structurally related compounds. Many analytical methods including gas chromatography (GC), GC-mass spectrometry (GC-MS), HPLC-ultraviolet detection (HPLC-UV), HPLC-fluorescence detection and HPLC-MS have been described for the measurements of lignans in plants.^[17,18] Though HPLC-UV is regularly used, this method is less sensitive because of poor detection nature of the prisms used in UV detector. HPLC-fluorescence detection, HPLC-MS and GC-MS are highly sensitive

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DOI: 10.5530/pj.2014.2.8

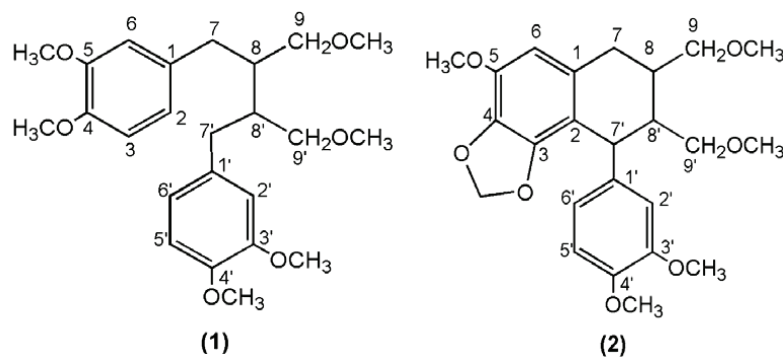


Figure 1. Chemical structures of phyllanthin (1) and hypophyllanthin (2).

with very low levels of detection of sample in plasma, but these methods are very expensive because of high cost of the equipment and laborious sample processing methods. These methods also include additional steps like prefiltration and derivatization of sample before injection. It is not possible for small research groups and academic departments to purchase and maintain these expensive instruments. Hence, there is need for the development of simple, reliable, sensitive and cost effective method for the analysis of bioactive lignans. In the present paper, reverse phase (RP) HPLC method with photo-diode-array (PDA) detection was developed for systematic identification and pharmacokinetic studies of the two bioactive lignans, phyllanthin and hypophyllanthin in solvent system and also in blood plasma.

EXPERIMENTAL

Chemicals and reagents

Silica gel 60–120 mesh was purchased from ACME Synthetic Chemicals (Mumbai, India), HPLC grade methanol was purchased from Merck (Germany). Deionized water for HPLC was obtained from Milli-Q instrument (Millipore, USA). EDTA centrifugation tubes were obtained from CML Biotech (P) Ltd (India).

Extraction and isolation

The leaves of *P. amarus* were collected from Paderu, Visakhapatnam District, Andhra Pradesh and authenticated by Dr. M. Venkayya, Taxonomist. Voucher specimen (BG/PMK/PA-10-10) was deposited in the herbarium, College of Pharmaceutical Sciences, Andhra University. Freshly collected aerial parts of the plant were shade dried and pulverized. The powdered material (5kg) was then subjected to successive soxhlet extraction with hexane, ethyl acetate and methanol. Solvent thus obtained

was separately concentrated under vacuum at 40°C by using rotary evaporator (Buchi, Switzerland). 25g of hexane extract was then fractionated over a column (100cm length × 35mm diameter) of silica gel (60–120 mesh size). Gradient elution was done in the following sequence, hexane (100, v/v) → hexane-ethylacetate (95:5, v/v) → hexane-ethylacetate (90:10, v/v). Fractions (Fr150–189) collected for hexane-ethylacetate (90:10 v/v) showed the presence of lignans on TLC and these fractions were pooled together and concentrated. The lignan fraction was subjected to preparative separation using RP-HPLC (Waters Delta Prep-PDA, USA) for obtaining the pure (>95%) phyllanthin and hypophyllanthin.^[15,16]

Instrumentation

HPLC system consists of a quaternary gradient HPLC (Waters Delta Prep HPLC system, USA) with a rheodyne injector for loading the sample. A reversed phase C-18 column (250m × 4.0mm; Waters associates, USA) was used with the following analytical conditions: mobile phase of methanol-water (66:34, v/v) with a flowrate of 1 ml/min at room temperature (27°C). Sample injection volume was 20 μl. Chromatograms were recorded at 199–400nm range using a photodiode array detector (Waters PDA 2998). HPLC system was equipped with EMPOWER 2 software (Waters, Milford, MA, USA) for data acquisition and processing.

Animals

Healthy Sprague Dawley rats of either sex weighing about 220–250gm were supplied by National institute of nutrition, Hyderabad. Animals were acclimatized under standard environment conditions (23 ± 2°C, 55 ± 5% relative humidity, 12h light/dark cycle) for one week. Pharmacokinetic studies were carried out in NAIP/ICAR Project laboratory, University college of Pharmaceutical Sciences, Andhra University and were approved

by institutional animal ethics committee (Regd. No. 516/PO/c/01/CPCSEA) for experimentation on animals.

Preparation of stock solutions and calibration standards

Stock solutions (1mg/ml) of compounds phyllanthin and hypophyllanthin were prepared in methanol (HPLC grade) and were further diluted with methanol to prepare different concentrations of their standard solutions. Calibration curves were established on twelve data points covering a concentration range of 0.5 to 100 μ g/ml for both compounds. Aliquots (20 μ l) were used for the HPLC injections. Linear regression of peak area of compounds of interest versus compound concentration was performed in order to estimate the slope, intercept and correlation coefficient of each calibration curve.

Plasma sample preparation

Blood samples were collected into micro-centrifuge tubes containing approximately 10mg heparin; centrifuged at 4000 \times g for 5 min at 15°C. To 125 μ l of plasma, 25 μ l of internal standard (carbamazepine stock solution 10 μ g/ml in methanol) was added and vortexed (Vortex mixer, Genei, Mumbai) for 60 seconds. Then 500 μ l of methanol was added to precipitate proteins and vortexed for 5 min and centrifuged at 5000 \times g for 10 min. Supernatant was taken and dried in vacuum oven at 40°C. Dried samples were then redispersed in 100 μ l methanol and vortexed.^[19] Supernatant was transferred in to a micro-centrifuge tube and from this 20 μ l was injected for HPLC analysis.

Assay validation

Accuracy and precision

Samples were analyzed against calibration curves. Meanwhile, standard deviation and relative standard deviation (RSD) were calculated from samples and used to estimate their intra and inter-day precision. Accuracy was assessed by comparison of calculated mean concentrations with their known concentrations. The intra-day accuracy and precision was determined for each compound at four concentrations with six replicates each carried out in a single day, whilst the inter-day values were carried out over 6 consecutive days. The accuracy was expressed in terms of recovery, and calculated by multiplying the ratio of measured drug concentration to its theoretical concentration with 100, so as to give percent recovery, whereas precision was expressed as coefficient of variation.

Recovery studies

The recovery efficiency was determined by adding measured amounts (2, 6, 10 and 40 μ g) of phyllanthin and hypophyllanthin to their known concentrations (10 μ g). Accuracy was expressed in terms of the recovery and calculated by multiplying the ratio of measured drug concentration to its theoretical concentration with 100, so as to give percent recovery. The study was replicated 6 times and values were expressed as mean \pm standard deviation (SD).

Limit of detection (LOD), limit of quantification (LOQ) and linearity

LOD was defined as the lowest concentration that analytical system can reliably differentiate from the background level, whilst LOQ was defined as lowest quantifiable concentration that can be measured with a stated level of confidence. Stock (1mg/ml) solutions of phyllanthin and hypophyllanthin were prepared in pooled blank rat plasma. LOD and LOQ were determined by injections of successive two-fold dilution of stock solutions in blank rat plasma. Standard calibration curves were constructed as plots of mean peak area ratio against the corresponding concentration at a range of 0.1–100 μ g/ml for both the lignans. Linearity of the curve was evaluated by linear regression analysis.

Pharmacokinetic study

Animals were divided into six groups of 4 each. First three groups (Groups I–III) received phyllanthin orally at doses of 2.5, 5 and 10mg/kg, respectively. The next three groups (Groups IV–VI) received hypophyllanthin orally at doses of 2.5, 5 and 10mg/kg, respectively. Lignans were given orally in aqueous solution of Tween 20 and the volume was made to 5ml/kg. Blood samples (0.4ml) were withdrawn from retro-orbital plexus at 0, 30 min, 1, 2, 4, 8, 12, 24 and 36 h after oral administration and were collected into micro centrifuge tubes containing approximately 10 mg heparin; centrifuged at 4000 \times g for 5 min at 15°C and resulting plasma was kept at –20°C before HPLC analysis.

Data analysis

The mean plasma concentration versus time data was analyzed by non-compartmental extravascular method and the following pharmacokinetic parameters were estimated: Peak plasma concentration (C_{max}) and time to reach peak concentration (T_{max}), elimination rate constant (K_e), terminal elimination half-life ($t_{1/2}$), area under

the plasma concentration–time curve (AUC), volume of distribution of terminal phase (V_d) and total body clearance (Cl) were calculated by taking plasma drug concentration (ng/ml) on y-axis and time (h) on x-axis using Kinetica software (Thermo scientific, USA) by log-linear trapezoidal rule.

Statistical analysis

Pharmacokinetic parameters of the three doses after oral administration of phyllanthin and hypophyllanthin were compared by one-way analysis of variance (ANOVA) model, respectively. The results were expressed as mean \pm SD. Statistical significance was determined at the level of $P < 0.05$.

RESULTS AND DISCUSSION

Assay validation

The objective of this study was to develop a sensitive HPLC method for the analysis of phyllanthin and hypophyllanthin in solvent system and plasma samples using most commonly employed C–18 column with PDA detection. Retention times (R_t) for phyllanthin and hypophyllanthin were observed as 27.69 and 24.16 minutes. No interfering peaks were found in the chromatogram.

Figs. 2A and 2B demonstrate the chromatograms in which the analytes were eluted without interference. The recovery, within-day and between-day precision and accuracy for measurements of different lignan concentrations are depicted in Table 1. Calibration curves of the both compounds showed linearity in the range of 0.1–100 μ g/ml. The linear regression equation for calibration curves of phyllanthin and hypophyllanthin were $Y = 22601x + 25070$

and $Y = 16339x + 28982$, respectively. The intra and inter-day precisions of phyllanthin and hypophyllanthin were in the range of 1.97–39.89 and 1.969–39.91. In accuracy assessment, recovery for phyllanthin and hypophyllanthin was found to be 99.08–99.82% and 97.98–100.52%, respectively. Thus, this HPLC method is simple, sensitive, precise and highly accurate.

Pharmacokinetic study

Chromatograms of plasma sample from a rat after 1 h of administration of 5mg/kg of phyllanthin and hypophyllanthin spiked with carbamazepine (R_t -6.4 min) were shown in Figs. 2A and 2B, respectively. The total run time was 40 minutes.

Calibration curves over a concentration range of 0.25–100 μ g/ml was linear for phyllanthin and hypophyllanthin with regression equations of $Y = 0.539x + 0.017$ and $Y = 0.450x - 0.087$, respectively. The correlation values of both compounds was greater than 0.999. Because of large difference in plasma level of lignans, a wider analytical range was used in present study to cover the entire range of expected concentrations. LOD and LOQ values of the tested lignans were approximately 56.14 ng/ml and 169.99ng/ml for phyllanthin; 56.04ng/ml and 169.82ng/ml for hypophyllanthin at signal to noise ratio of 5:1.

The reported HPLC–MS method for analysis of plasma lignans has limits of detection between 0.021–0.2ng/ml.^[20] It was reported that analysis of lignans in biological fluids by GC–MS method had LOD values between 0.04 and 10nM, it was less than 5nM for HPLC–MS method. Vikneswaran and Chan reported the HPLC–fluorescence method with detection limits between 2.91–14.19nM for lignan analysis in plasma.^[21] Furthermore, Nurmi et al reported a HPLC method using coulometric electrode

Table 1. Recovery, Intra-day and Inter-day precision values of phyllanthin and hypophyllanthin in solvent system.

| Drug concentration (μ g/ml) | Recovery | | Precision | |
|----------------------------------|--|--------------------------------------|-----------------------|----------------------|
| | Mean (\pm SD) amount (μ g) recovered (n=6) | Mean (\pm SD) % of recovery (n=6) | Intra-day (% CV), n=6 | Inter-day (%CV), n=6 |
| Phyllanthin | | | | |
| 2 | 1.98 \pm 0.14 | 99.08 \pm 0.38 | 1.97 (0.46) | 1.969 (0.27) |
| 6 | 5.95 \pm 0.11 | 99.11 \pm 1.78 | 6.02 (3.69) | 5.98 (1.51) |
| 10 | 9.94 \pm 0.028 | 99.34 \pm 0.28 | 9.91 (0.76) | 9.84 (1.17) |
| 40 | 39.92 \pm 0.67 | 99.82 \pm 0.17 | 39.89 (0.14) | 39.91 (0.52) |
| Hypophyllanthin | | | | |
| 2 | 1.959 \pm 0.11 | 97.98 \pm 0.56 | 1.95 (1.26) | 1.952 (1.12) |
| 6 | 6.03 \pm 0.19 | 100.52 \pm 1.44 | 5.84 (1.03) | 5.94 (0.52) |
| 10 | 9.94 \pm 0.042 | 99.36 \pm 0.42 | 9.94 (0.41) | 9.87 (0.71) |
| 40 | 39.76 \pm 0.27 | 99.41 \pm 0.67 | 39.77 (2.62) | 39.29 (2.5) |

array detection with detection limits between 1.9–3.9nM for lignan analysis in human urine.^[22] The present HPLC–PDA method has comparable detection and quantification limits.

The recovery, intra and inter-day precision and accuracy measurements of different concentrations are revealed in Table 2. The present method produced a satisfactory recovery of lignans phyllanthin and hypophyllanthin from 97.12–99.74% and 98.34–99.76% respectively, thus implying that deproteinization of plasma with methanol did not result in any substantial loss of chemical constituents. The intra-day and inter-day precision and accuracy were between 97.12–99.74 for phyllanthin and 98.34–99.76 for hypophyllanthin, respectively, indicating that the developed method was reliable and reproducible. Figs. 3A and 3B reveals mean plasma concentration versus time profiles up on oral administration of 2.5, 5 and 10mg/kg of phyllanthin and hypophyllanthin, respectively.

The present HPLC–PDA method makes it possible to measure lignan concentrations in rat plasma even up to 12h after oral administration. Pharmacokinetic parameters of lignans in rat plasma after oral administration are depicted in Table 3. Both lignans showed a rapid rise with a T_{max} of 1h followed by a gradual decline to 0 after 24h. Both compounds showed non-linear increase in $AUC_{0\rightarrow 24h}$ values with increase in their oral doses. Being

lipophilic (isolated from the hexane extract of *P.amarus*), dissolved lignans could penetrate the gastrointestinal tract more easily achieving their peak plasma concentration after 1h.

CONCLUSION

A simple, sensitive and validated HPLC–PDA detection method for the determination of phyllanthin and hypophyllanthin in solvent system and plasma has been developed. This method was accurate and precise for the quantitative analysis of lignans and it has additional advantages over known methods such as simple sample preparation, low detection and quantification limits. The developed method was successfully applied in the pharmacokinetic study of lignans in rats.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

ACKNOWLEDGEMENT

This work was funded by National Agricultural Innovation Project and Indian Council for Agricultural Research (NAIP/ICAR; Grant No.C4/40043101), Government of India.

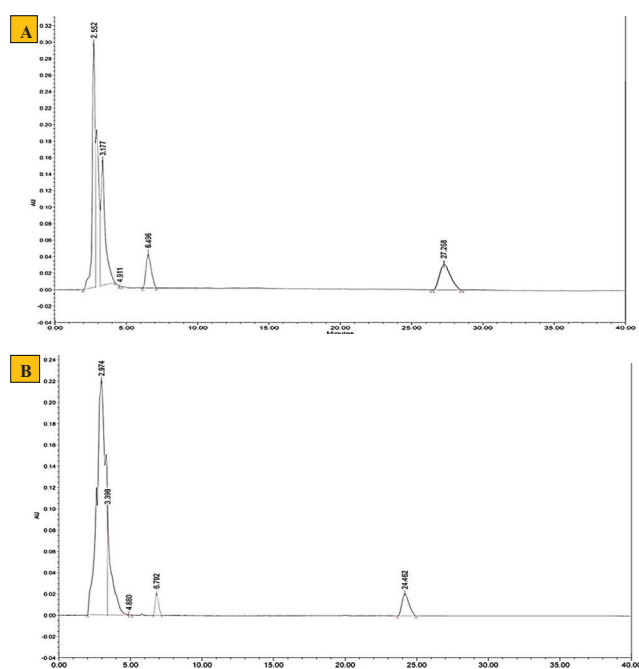


Figure 2A & B. HPLC chromatograms of rat plasma after administration of 5mg/kg of phyllanthin and hypophyllanthin.

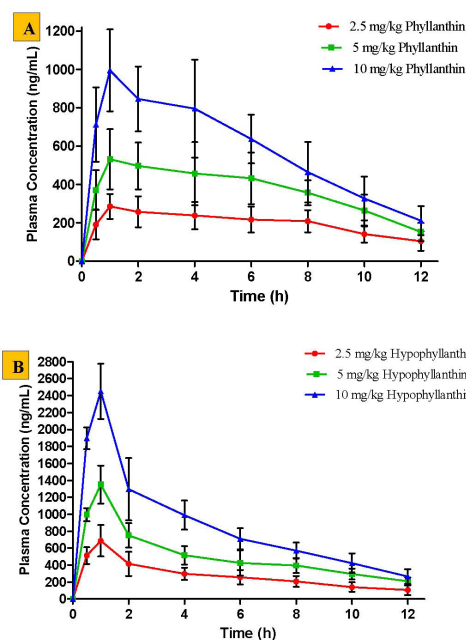


Figure 3A & B. Plasma concentration (mean \pm SD, n=6) versus time profiles after oral administration of phyllanthin and hypophyllanthin at doses of 2.5, 5 and 10mg/kg to rats.

Table 2. Recovery, Intra-day and Inter-day precision values of phyllanthin and hypophyllanthin in rat plasma.

| Drug concentration (µg/ml) | Recovery | | Precision | |
|----------------------------|---|---------------------------------|-----------------------|----------------------|
| | Mean (± SD) amount (µg) recovered (n=6) | Mean (± SD) % of recovery (n=6) | Intra-day (% CV), n=6 | Inter-day (%CV), n=6 |
| Phyllanthin | | | | |
| 2 | 1.956 ± 0.25 | 97.12 ± 1.26 | 1.96 (0.458) | 1.945 (0.97) |
| 6 | 5.92 ± 0.14 | 98.56 ± 2.45 | 5.94 (1.79) | 5.88 (3.31) |
| 10 | 9.84 ± 0.15 | 98.42 ± 1.16 | 9.68 (3.8) | 9.86 (0.81) |
| 40 | 39.89 ± 0.27 | 99.74 ± 0.17 | 39.79 (0.51) | 39.89 (0.18) |
| Hypophyllanthin | | | | |
| 2 | 1.94 ± 0.22 | 98.34 ± 1.04 | 1.96 (0.92) | 1.962 (1.46) |
| 6 | 5.91 ± 0.18 | 98.38 ± 0.69 | 5.91 (0.25) | 5.86 (0.41) |
| 10 | 9.84 ± 0.14 | 98.32 ± 1.31 | 9.72 (3.51) | 9.73 (1.98) |
| 40 | 39.89 ± 0.24 | 99.76 ± 0.15 | 39.62 (1.25) | 39.84 (0.97) |

Table 3. Pharmacokinetic parameters (mean ± SD, n=6) after oral administration of phyllanthin and hypophyllanthin to rats.

| Parameters | Phyllanthin | | | Hypophyllanthin | | |
|------------------------------------|--------------------------|-------------|-------------|--------------------------|-------------|--------------|
| | 2.5 mg/kg | 5 mg/kg | 10 mg/kg | 2.5 mg/kg | 5 mg/kg | 10 mg/kg |
| C _{max} (µg/ml) | 0.28 ± 0.06 | 0.53 ± 0.16 | 0.98 ± 0.22 | 0.68 ± 0.76 | 1.35 ± 0.23 | 2.45 ± 0.33 |
| T _{max} (h) | 1 ± 0.09 ^a | 1 ± 0.22 | 1 ± 0.25 | 1 ± 0.14 ^a | 1 ± 0.25 | 1 ± 0.18 |
| AUC (µg h/ml) | 2.81 ± 0.15 | 5.24 ± 0.26 | 8.09 ± 0.19 | 3.89 ± 0.11 | 7.37 ± 0.17 | 12.17 ± 0.27 |
| k _{el} (h ⁻¹) | 0.22 ± 0.03 ^a | 0.22 ± 0.07 | 0.19 ± 0.08 | 0.17 ± 0.02 ^a | 0.16 ± 0.05 | 0.14 ± 0.04 |
| t _{1/2} (h) | 3.22 ± 0.28 ^a | 3.27 ± 0.14 | 3.53 ± 0.26 | 4.07 ± 0.35 ^a | 4.34 ± 0.17 | 4.54 ± 0.26 |
| Cl (L/h) | 0.87 ± 0.11 ^a | 0.96 ± 0.24 | 1.24 ± 0.21 | 0.64 ± 0.14 ^a | 0.68 ± 0.22 | 0.82 ± 0.25 |
| Vz (L) | 4.15 ± 0.94 ^a | 4.49 ± 1.15 | 6.28 ± 1.67 | 3.78 ± 1.05 ^a | 4.24 ± 0.86 | 5.37 ± 1.26 |

^a Values were significantly different from 5 and 10mg/kg (P<0.05).

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