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# Development and Validation of HPTLC Method for Simultaneous Determination of Wedelolactone and Phyllanthin in Hepasuport Tablet

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## ABSTRACT

*A new and simple HPTLC method was developed and validated for the simultaneous quantitative determination of Wedelolactone and Phyllanthin in hepatoprotective Hepasuport tablet. TLC aluminium plates precoated with silica gel 60F-254 (0.2 mm thickness) were used. The samples were dissolve in methanol and linear ascending development was carried out in twin trough glass chamber saturated with mobile phase Toluene: Ethyl acetate: Formic acid (5.0:4.0:1.0, v/v/v) and densitometric determination of these compounds was carried out by TLC scanner (CAMAG) at 254 nm in reflectance/absorbance mode. The $R_f$ value of wedelolactone and phyllanthin was found to be 0.56 ± 0.02 and 0.65 ± 0.03 respectively. The method was validated with respect to specificity, linearity, accuracy, precision, ruggedness and robustness as per the International Conference on Harmonization (ICH) guidelines. Results found to be linear in the concentration range of 600ng to 1600 ng with $r^2$ = 0.998 and 0.995 for wedelolactone and phyllanthin, respectively. The percent recoveries were found to be 100.06 and 99.35 for wedelolactone and phyllanthin respectively. The method can be used for routine analysis of these phytoconstituents in tablet dosage forms in quality control laboratories.*

**Keywords:** Phyllanthin, simultaneous HPTLC, validation, wedelolactone.

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## INTRODUCTION

Medicinal plants commonly included in Ayurveda for liver ailments have drawn much attention as there is no reliable hepatoprotective drug available in modern medicine [**1**]. Liver is the most important organ of metabolism and excretion. About 20,000 deaths occur every year due to liver diseases [**2**]. The management of liver diseases is still a challenge to the modern medicine [**3**].

Standardization of herbals is a difficult process since the herbal contains complex mixtures of different components or mixtures of herbals are used at times as prevalent in different systems of medicines such as Ayurveda [**4**]. Over the past decade HPTLC has been successfully used in the analysis of pharmaceuticals, plant constituents, and biomacromolecules. Several samples can be run simultaneously using a small quantity of mobile phase, thus lowering analysis time and cost per analysis [**5**]. This includes developing TLC fingerprint profiles and estimation of chemical markers and biomarkers [**6**]. Now day's new multicomponent formulations in market increasing with alarm rate which have better synergetic effect it is very essential that two or more number of drugs should be estimated simultaneously [**7**].

Wedelolactone is a coumestan derivative obtained from herb *Eclipta alba* Linn., Hassk (Family-Compositae) having a potent and selective 5- lipoxygenase inhibitor with an $IC_{50}$ of 2.5 µM and it does so by an oxygen radical scavenging mechanism [**8**].

Phyllanthin is a lignan compound obtained from herb *Phyllanthus niruri* (family-Euphorbiaceae) reported to inactivate Hepatitis-B both *in vitro* and *in vivo* [**9**].

Literature survey reveals TLC, HPTLC, HPLC, spectrophotometry and spectrofluorometric methods were used for determination of wedelolactone as single and in combination with other phytoconstituents [**10-18**]. TLC, HPTLC, HPLC, RP-LC, and derivative spectrophotometry methods were used for determination of phyllanthin as single and in combination with other phytoconstituents [**19-22**].

No reports were found for the simultaneous estimation of wedelolactone and phyllanthin by HPTLC. This paper describes a simple, accurate and validated method for simultaneous quantification of these compounds in tablet dosage form. The proposed method was optimized and validated as per the International Conference on Harmonization (ICH) guidelines [**23**].

## MATERIALS AND METHODS

**Materials:**

Reference standard wedelolactone and phyllanthin were purchased from Sigma Aldrich, (Germany) and SPIC Pvt. Ltd. (India) respectively. Analytical grade Toluene, Ethyl acetate, Formic acid and Methanol were purchased from S. D. Fine Chem. Ltd. (India).

**Instrumentation and Chromatographic Conditions:**

The samples were spotted in the form of bands of width 6 mm with a Camag 100 microlitre sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminum plate 60F–254 plates, (20 cm × 10 cm with 250 µm thickness; E. Merck, Darmstadt, Germany) using a Camag Linomat V (Switzerland) sample applicator. The plates were prewashed with methanol and activated at $110^0$C for 5 min prior to chromatography. A constant application rate 0.1µl/s was used and the space between two bands was 10 mm. The slit dimension was kept at 4 mm × 0.45 mm and the scanning speed was 20 mm/s. The monochromator bandwidth was set at 20 nm, each track was scanned three times and baseline correction was used. The mobile phase was toluene: ethyl acetate: formic acid (5.0:4.0:1.0 v/v/v). Linear ascending development was carried

out in a 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase. The optimized chamber saturation time for the mobile phase was 30 min at room temperature ($25^0$C ± 2) at relative humidity 60 % ± 5. The length of each chromatogram run was 8 cm. Following the development, the TLC plates were dried in a current of air with the help of an air dryer in a wooden chamber with adequate ventilation. Densitometric scanning was performed using a Camag TLC scanner III in the reflectance/absorbance mode at 254 nm and operated by CATS IV CAMAG software. The source of radiation used was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compounds were determined from the intensity of the diffused light. Evaluation was by peak areas with linear regression. The amount of wedelolactone and phyllanthin was computed from peak areas.

**Preparation of Standard Stock Solution:**
Accurately weighed 1mg of wedelolactone and phyllanthin was dissolved in 2 ml methanol, sonicate and diluted with methanol up to 10 ml (100µg/ml).

**Preparation of Hepasuport Tablet Solution:**
Hepasuport tablets powder (5 g) was refluxed with methanol and volume was made up to 10 ml (500 mg/ml). The solution was filtered using Whatman paper No. 1. The 1 ml solution pipette out from stock solution was further diluted up to 5 ml with methanol to get final concentration 100 µg/µl.

**Validation of Method:**
The method was validated for linearity, specificity, accuracy, recovery, intra-day and inter-day precision, ruggedness, robustness, LOD and LOQ in accordance with ICH guidelines [**23**].

**Preparation of Calibration Curve:**
A mixture of standard solution of wedelolactone and phyllanthin in methanol (100 µg/ml or 100 ng/µl) was applied in 6, 8, 10, 12, 14 and 16 µl, on the TLC plate to prepare linear calibration curve.

**Specificity:**
The specificity of method was ascertained by spotting solutions of standard wedelolactone, phyllanthin and sample Hepasuport tablet solution on TLC plate in triplicate and run. The spots for wedelolactone and phyllanthin in the samples of Hepasuport tablet were confirmed by comparing the $R_f$ values and spectrum with standards. The results are shown in Table 1.

**Recovery Studies:**
The accuracy was studied by the standard addition technique. Three different levels of standard were added to the previously analyzed samples, each level being repeated thrice. The results of recovery studies were expressed as percent recovery. The results are shown in Table 2.

**Precision:**
All the solutions were analyzed on the same day in order to record any intraday variations in the results. For Inter day variation study, three different concentrations of the mixed standard solutions in linearity range were analyzed on three consecutive days. The repeatability of sample

application and measurement of the peak area was expressed in terms of %RSD. The %RSD was found to be less than 2.0 in all cases indicate no significant variations in the analysis of wedelolactone and phyllanthin at the concentration of 600, 800, 1000 ng/spot.

**Robustness Studies:**
The estimations were performed by introducing variations in the mobile phase distance development and saturation time in development chamber; the effects on the results were examined. Mobile phase distance was changed by ±5 mm. The saturation time of mobile phase in the chamber was varied by ±5 min. The %RSD was found to be less than 1.0 in all cases indicates no significant variations in the analysis of wedelolactone and phyllanthin at the concentration of 600 ng/spot.

**Ruggedness Studies:**
The HPTLC method was evaluated by carrying out the analysis of standard solutions by two analysts using same operational and environmental conditions, the %RSD was found to be less than 1.0 in the analysis of wedelolactone and phyllanthin at the concentration of 600 ng/spot.

**Limit of detection (LOD) and Limit of quantification (LOQ):**
The LOD and LOQ were calculated by using the values of slopes and intercepts of the calibration curves.

**Table 1: Estimation of Wedelolactone and Phyllanthin in Hepasuport tablet**

| Component | Amount taken(µg/spot) | Peak area Mean ± SD | % RSD | Amount Found (ng) | % Amount Found |
|---|---|---|---|---|---|
| Wedelolactone | 500 | 5036.5±33.48 | 0.66 | 1714.5 | 0.34 |
| Phyllanthin | 500 | 440.3±4.60 | 1.04 | 753.8 | 0.15 |

**Table 2: Recovery Studies of Wedelolactone and Phyllanthin**

| Drug | Amount taken (ng/band) | Amount taken (ng/band) | Total amount found (ng/band) | % Recovery | % RSD |
|---|---|---|---|---|---|
| Wedelolactone | 680 | 340 | 1022.3 | 100.2 | 100.06 |
| | 680 | 680 | 1359.8 | 99.98 | |
| | 680 | 1020 | 1700.4 | 100.02 | |
| Phyllanthin | 450 | 225 | 675.01 | 100.0 | 99.35 |
| | 450 | 450 | 897.8 | 99.75 | |
| | 450 | 675 | 1105.9 | 39.30 | |

## RESULTS AND DISCUSSION

The mixture of different mobile phases were tried and finally Toluene: Ethyl acetate: Formic acid (5.0:4.0:1.0, v/v/v) was selected, which gave good resolution. Representative densitogram of mixed standard solution of wedelolactone and phyllanthin is shown in Figure 1.

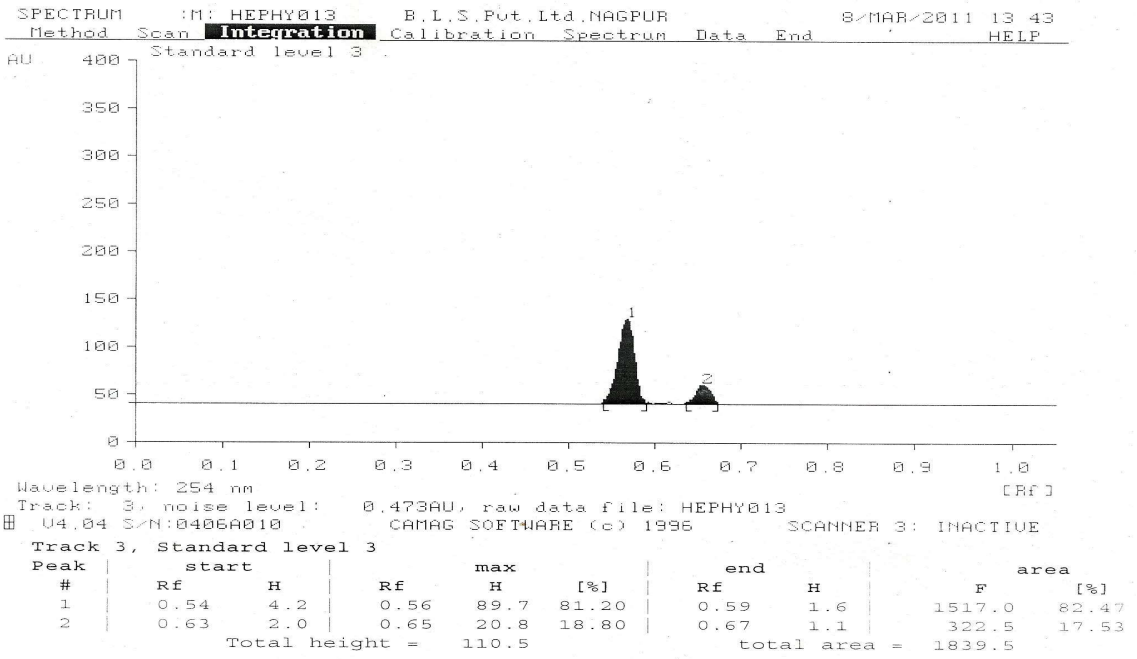


**Figure 1: Representative densitogram of standard wedelolactone (600 ng/spot): peak-1 (Rf 0.56±0.03) and phyllanthin (600ng/spot) peak-2 (Rf 0.65±0.02)**

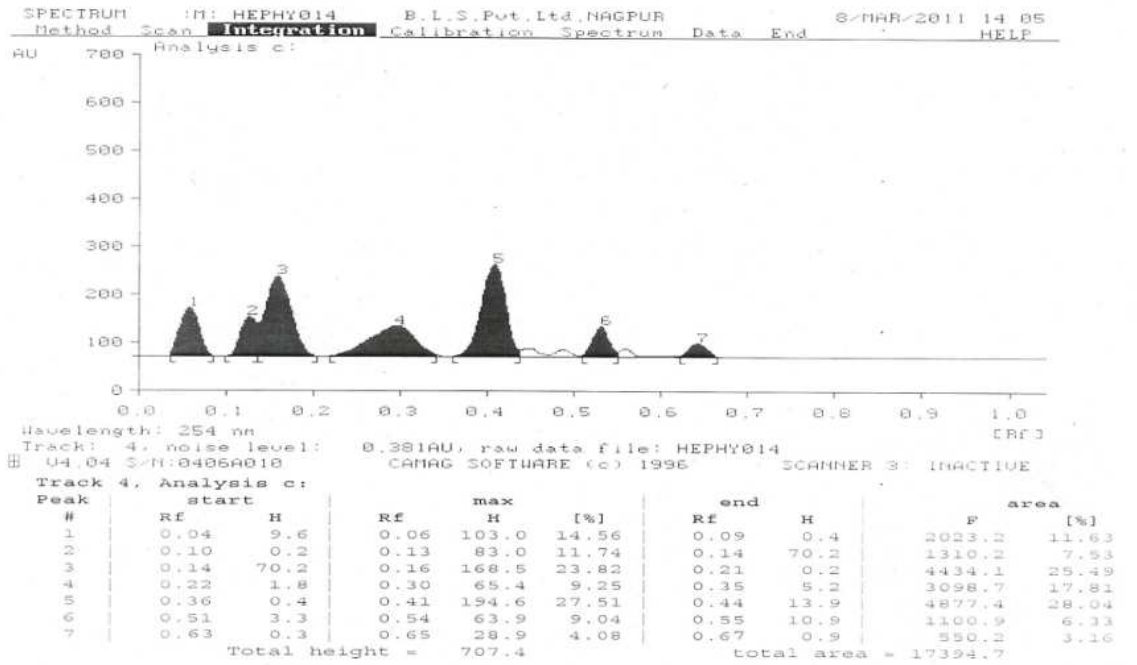


**Figure 2: Estimation of wedelolactone and phyllanthin in Hepasuport tablet (500µg/spot) peak 1-7 belongs to components in Hepasuport tablet in which peak 6 is of wedelolactone and peak 7 is of phyllanthin (Rf 0.54±0.03 and 0.65±0.02 respectively).**

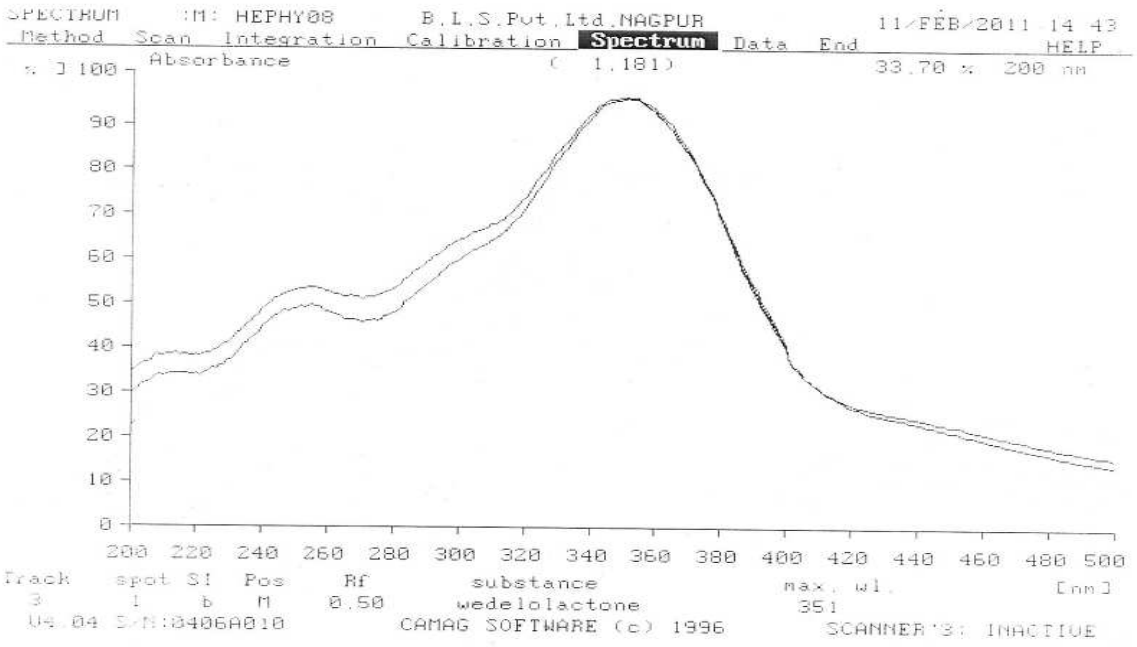


**Figure 2: UV Spectrum of Standard Wedelolactone and Hepasuport Tablet**

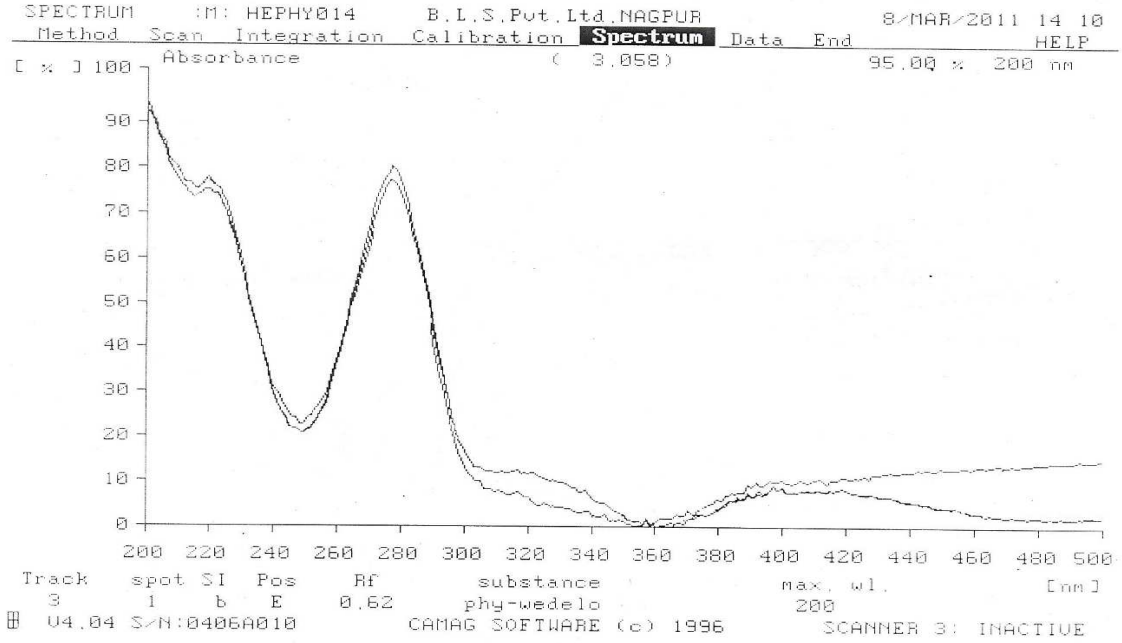


**Figure 3: UV Spectrum of Standard Phyllanthin and Hepasuport Tablet**

The $R_f$ value of wedelolactone and phyllanthin was found to be 0.56 ± 0.02 and 0.65 ± 0.03 respectively. Calibration curve plots of wedelolactone and phyllanthin peak area against concentration were linear in the range 600-1600 ng/spot, with linear equations Y= 3.119X-311.1

for wedelolactone and Y= 0.459X+94.27 for phyllanthin. The specificity of method is shown in Figure 2, 3 and 4.

The percent recoveries were found to be 100.06 and 99.35 for wedelolactone and phyllanthin respectively. Robustness of the method checked after deliberate alterations of the analytical parameters showed that areas of peaks of interest remained unaffected by small changes of the operational parameters (%RSD < 2).

The summary of validation parameters of proposed method are given in Table 3.

**Table 3: Validation Study Data of Proposed Method**

| Parameters | Wedelolactone | Phyllanthin |
|---|---|---|
| Linearity range (ng/spot) | 600- 1600 | 600- 1600 |
| Correlation coefficient (r) | 0.998 | 0.995 |
| Accuracy (% Recovery) | 100.06% | 99.35% |
| Intra-day Precision | 0.89 | 1.04 |
| Inter-day Precision | 0.95 | 1.13 |
| Robustness (Development distance) | 0.67 | 0.79 |
| Robustness (Saturation time) | 0.89 | 0.83 |
| Ruggedness | 0.87 | 0.89 |
| LOD | 16.87 | 51.13 |
| LOQ | 44.71 | 135.51 |

## CONCLUSION

The validated HPTLC method employed proved to be simple, fast, accurate, precise and robust thus can be used for routine analysis of wedelolactone and phyllanthin in tablet dosage form.

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