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# Development and Validation of HPTLC Method for Simultaneous Determination of Reserpine and Arjunolic acid in Tensowert Tablet

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## ABSTRACT

*Simultaneous quantitative estimation of two biologically active compounds reserpine and arjunolic acid in Tensowert tablet was performed using high-performance thin-layer chromatography. TLC aluminium plates precoated with silica gel 60F-254 (0.2 mm thickness were used. The sample were dissolve in methanol and linear ascending development was carried out in twin trough glass chamber saturated with mobile phase Toluene:Ethyl-acetate:Diethyl-amine:Glacial acetic acid (6.5:5.0:1.5:0.5 v/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 reserpine and arjunolic acid was found to be 0.42±0.03 and 0.14±0.02, respectively. Linearity was found to be in the concentration range of 200 ng to 1600 ng for both reserpine and arjunolic acid. The linear regression data for the calibration plots showed a good linear relationship with $r^2$=0.998 and 0.995 for reserpine and arjunolic acid, respectively. According to the ICH guideline the method was validate for accuracy, precision, recovery, robustness and ruggedness. The reserpine and arjunolic acid contents quantified from herbal formulation (Tensowert tablet) were found well within limits. Statistical analysis of the data showed that the method is reproducible and selective for the estimation of reserpine and arjunolic acid.*

**Key words**: HPTLC, reserpine, arjunolic acid, *Rauwolfia serpentina*, *Terminalia arjuna*.

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## INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death worldwide. Hypertension is the most common cardiovascular disease and a major public health problem in both developed and developing countries. Plant based drugs provide outstanding contribution to modern therapeutics; for example: reserpine isolated from *Rauwolfia serpentina* in 1953 was revolutionary event in

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the treatment of hypertension and lowering blood pressure [1]. Arjunolic acid isolated earlier from *Terminalia arjuna* has been widely used in Indian system of medicine for cardiac ailments [2]. Literature survey revealed that HPLC [3], UV [4], IR, NMR spectrometry, fluorescence spectrometry [5] methods reported for the estimation of reserpine and HPLC[6] , UV, NMR, mass spectrometry[7] reported for the estimation of arjunolic acid. HPTLC for reserpine [8] and TLC for arjunolic acid [9] were also developed. The objective of the present study was to developed simultaneous quantitative determination of reserpine and arjunolic acid, by HPTLC.

## MATERIALS AND METHODS

### 2.1 Drugs and Chemicals:
Reference standard reserpine and arjunolic acid were obtained from Natural Remedies Pvt. Ltd. Bangalore and Spice Pvt. Ltd. Chennai, respectively. Analytical grade of Toluene, Ethyl acetate, Diethyl amine, Glacial acetic acid and Methanol were purchased from Merck Chemical, Mumbai. Stationary phase was pre-coated silica gel aluminium plate 60F-254 was obtained from E. Merck (Germany). The aqueous extract of *R. serpentina* and *T. arjuna* were procured from Konarck Herbal Ltd. Mumbai.

### 2.2 Standard and sample preparation:
### 2.2.1 Sample preparation of *R. serpentina* and *T. arjuna* extracts:
Accurately weighed 100 mg of *R. serpentina* and *T .arjuna* extracts dissolved in 8 ml methanol and refluxed for 30 min. The solution was filtered through Whatman filter paper no. 1 and diluted with methanol up to 10 ml (10 mg/ml).

### 2.2.2 Preparation of test solution of Tensowert tablet:
Tensowert tablets powder (1 g) was refluxed with methanol and volume was made up to 10 ml (100 mg/ml). Pipette out 1 ml from the stock solution and diluted with methanol up to 5 ml (20 mg/ml).

### 2.2.3 Preparation of standard stock solution:
Accurately weighed 1mg of reserpine and arjunolic acid was dissolved in 2 ml methanol, sonicate and diluted with methanol up to 10 ml (100µg/ml).

### 2.3 Chromatographic condition:
The samples were spotted in the form of bands, width 6 mm with a Camag 100 microlitre sample (Hamilton, Bonaduz, Switzerland) syringe on silica gel precoated 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: diethyl amine: glacial acetic acid (6.5: 5.0:1.5:0.5 v/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

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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 reserpine and arjunolic acid was computed from peak areas.

### 2.4 Calibration curve:

A mixture of standard solution of reserpine and arjunolic acid in methanol (100 µg/ml or 100 ng/µl) was applied in 2, 4, 6, 8, 10, 12, 14 and 16 µl, on the TLC plate to prepare linear calibration curve.
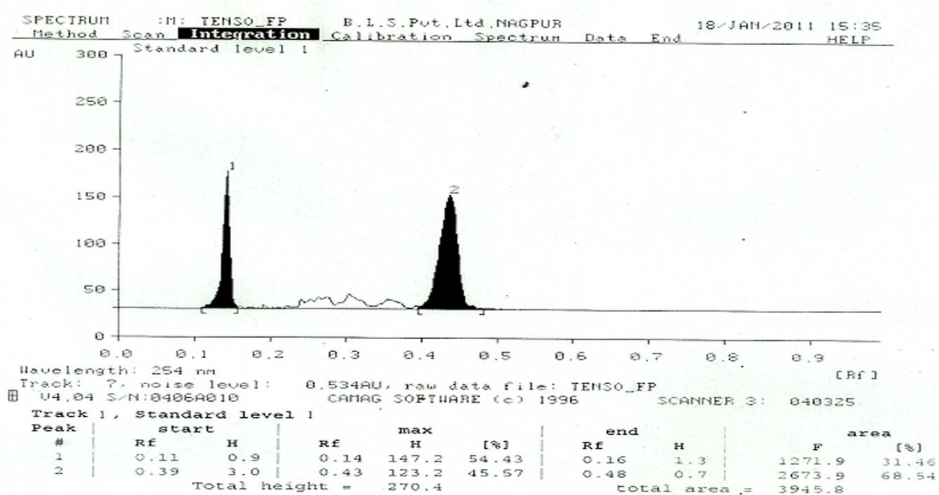


**Fig.1: Chromatogram of arjunolic acid (1200 ng/spot): peak-1 ($R_f$ 0.14±0.03) and reserpine (1200ng/spot) peak-2 ($R_f$ 0.42±0.03), mobile phase; toluene: ethyl acetate: diethyl-amine: glacial acetic acid (6.5: 5.0: 1.5: 0.5 v/v/v/v)**

## RESULTS AND DISCUSSION

### 3.1 Mobile phase development:

The mixtures of several mobile phases were tried to separate reserpine and arjunolic acid. The solvent system used toluene: ethyl acetate: diethyl-amine: glacial acetic acid (6.5:5.0:1.5:0.5) was selected for estimation of reserpine and arjunolic acid, which gave good resolution. Figure 1 is showing chromatographic separation of reserpine and arjunolic acid at $R_f$ 0.42±0.03 and 0.14±0.02 respectively. The absorption spectrum of reserpine and arjunolic acid is shown in Figure 2 and 3. The wavelength 254 nm was used for quantification of sample.
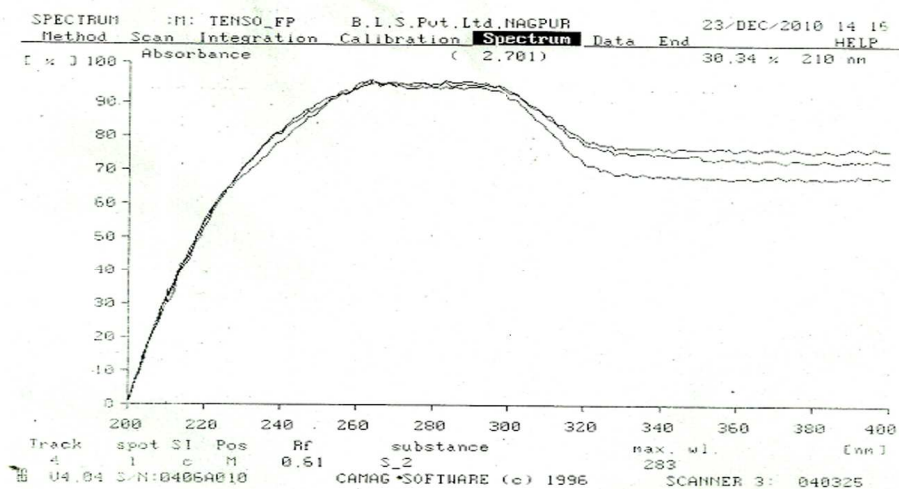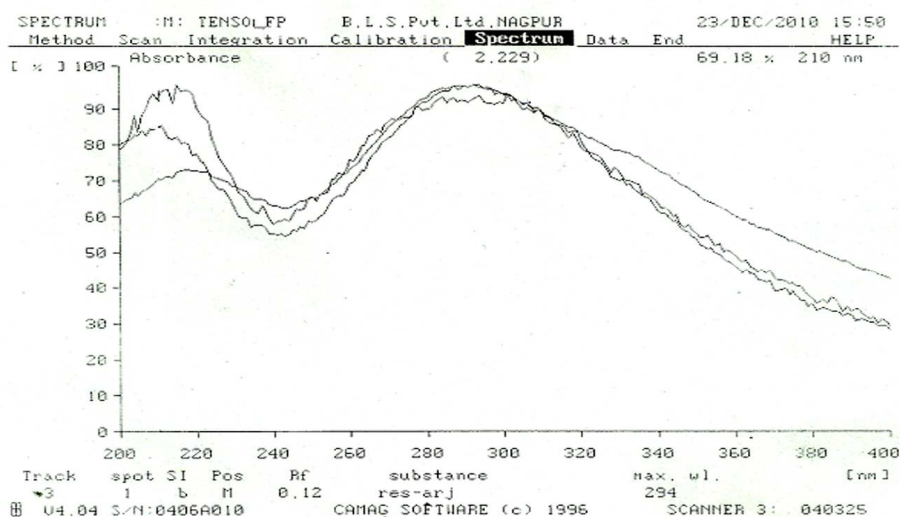
**Fig. 2: Absorption spectra of reserpine**



**Fig. 3: Absorption spectra of arjunolic acid**

## 3.2 Method validation:
### 3.2.1 Specificity:
The specificity of method was ascertained by standard reserpine, arjunolic acid and sample Tensowert tablet. The solutions of standard reserpine, arjunolic acid and samples Tensowert tablet were spotted on TLC plate in triplicate and run. The spots for reserpine and arjunolic acid in the samples were confirmed by comparing the $R_f$ values and spectrum with standards. The validation parameters for the proposed method are shown in Table 1.

### 3.2.2 Linearity:
Calibration curve plots of reserpine and arjunolic acid peak area against concentration were linear in the range 200-1600 ng/spot. The calibration lines were represented by linear equation Y

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= 1.715 X–124.4 for reserpine and Y=0.813X+9.517 for arjunolic acid. For these equations the correlation coefficient, $r^2$ was 0.998 for reserpine and 0.995 for arjunolic acid.

### 3.2.3 Limits of quantification and detection
The LOQ and LOD were calculated as 6.31 ng/spot and 19.12 ng/spot for reserpine; and 12.54 ng/spot and 38.00 ng/spot for arjunolic acid.

**Table1: Validation parameter for reserpine and arjunolic acid by HPTLC**

| Sr. No. | Parameter | RESERPINE | ARJUNOLIC ACID |
|---|---|---|---|
| 1 | Linearity range | 200 ng-1600 ng | 200 ng-1600 ng |
| 2 | Correlation coefficients | 0.998 | 0.995 |
| 3 | Régression equation (y = mx+c) | Y=1.715-124.4 | Y=0.813+9.517 |
| 3 | Accuracy(mean recovery) | 99.49% | 100.15% |
| 4 | Precision (RSD) | | |
| | Interday | 0.51 | 0.71 |
| | Intraday | 0.44 | 1.09 |
| 5 | LOD | 6.31ng | 12.54ng |
| 6 | LOQ | 19.12ng | 38.00ng |
| 7 | Ruggedness/Robustness(RSD) between two experiments | | |
| | Development distance | 0.38 | 0.70 |
| | Saturation time | 0.38 | 0.76 |
| | Analyst | 0.28 | 0.60 |
| 8 | specificity | Specific | Specific |

### 3.2.4 Precision:
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 reserpine and arjunolic acid at the concentration of 400, 600 and 800 ng/spot.

### 3.2.5 Robustness:
The estimations were performed by introducing variations in the mobile phase distance development; the effects on the results were examined. Mobile phase development distance was changed by ±5mm. 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 reserpine and arjunolic acid at the concentration of 600 ng/spot.

### 3.2.6 Ruggedness:
The estimation were performed by changing the analyst, the %RSD was found to be less than 1.0 in the analysis of reserpine and arjunolic acid at the concentration of 600 ng/spot.

### 3.2.7 Accuracy:
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 percentage recovery of reserpine and arjunolic acid was 99.49 and 100.15 in Tensowert tablet respectively, as shown in Table 2 and 3.

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**Table 2: Result and Statistical data for recovery study of reserpine**

| Sr. No. | Reserpine in sample (ng) | STD added (ng) | Total amount | Actual amount | % recovery | Mean |
|---------|--------------------------|----------------|--------------|---------------|------------|------|
| 1 | 582.8 | 291.4 | 874.2 | 869.07 | 99.41 | |
| 2 | 582.8 | 582.8 | 1165.6 | 1162.48 | 99.73 | 99.49% |
| 3 | 582.8 | 874.2 | 1457.8 | 1448.32 | 99.34 | |

**Table 3: Result and Statistical data for recovery study of arjunolic acid**

| Sr. No. | Arjunolic acid in sample (ng) | STD Added (ng) | Total added amount | Actual amount | % recovery | Mean |
|---------|-------------------------------|----------------|--------------------|---------------|------------|------|
| 1 | 307.5 | 153.7 | 461.2 | 458.13 | 99.33 | |
| 2 | 307.5 | 307.5 | 615.0 | 626.89 | 101.93 | 100.15% |
| 3 | 307.5 | 461.2 | 768.7 | 762.5 | 99.19 | |

### 3.2.8 Estimation of reserpine and arjunolic acid in *Rauwolfia serpentina*, *Terminalia arjuna* extracts and in Tensowert tablet:

The optimized solvent system was used for the estimation of reserpine in *Rauwolfia serpentina* extract, arjunolic acid in *Terminalia arjuna* extract and in Tensowert tablet. The resolution was good and components were observed at different $R_f$ value, as shown in Figure 4, 5 and 6 respectively. The method was used to determine the reserpine and arjunolic acid content in *Rauwolfia serpentina*, *Terminalia arjuna* extracts and in Tensowert tablet. The results are shown in Table 4, 5, 6. The low RSD values are indicate the high accuracy and precision of the method.
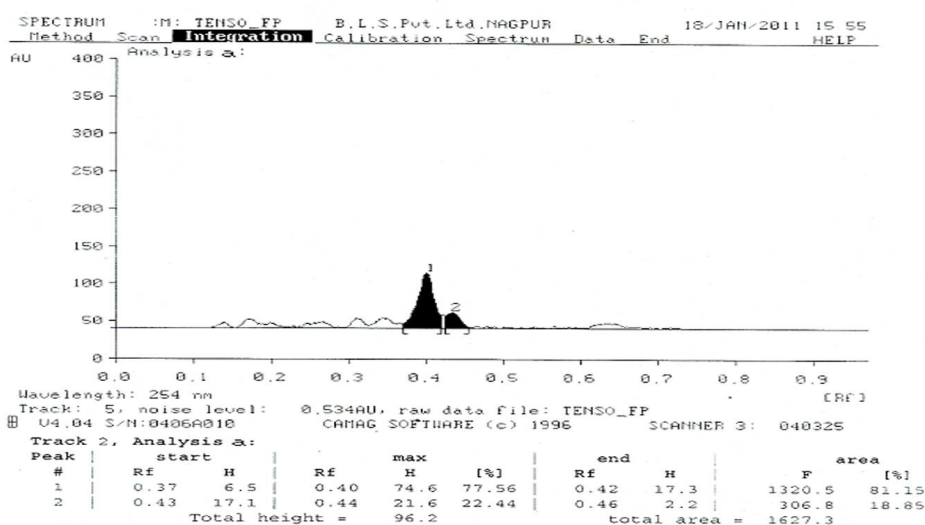


**Fig.4: Estimation of reserpine in *Rouwolfia serpentina* extract (100μg/spot) peak 1-2 belongs to components present in extract in which peak 1 is of reserpine ($R_f$ 0.40±0.03); mobile phase toluene: ethyl acetate: diethyl-amine: glacial acetic acid (6.5: 5.0: 1.5: 0.5 v/v/v/v)**
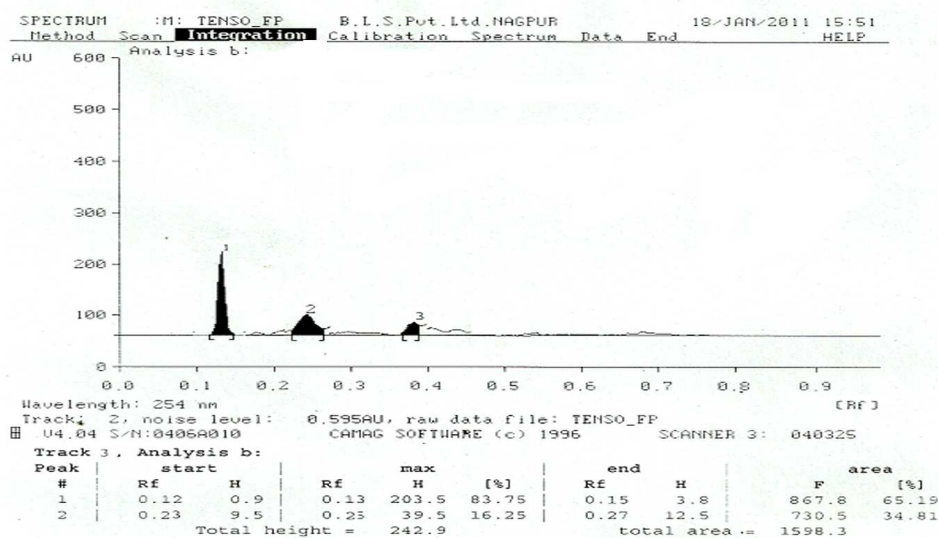
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**Fig.5: Estimation of arjunolic acid in *Terminalia arjuna* extract (100μg/spot) peak 1-3 belongs to components present in extract in which peak 1 is of arjunolic acid ($R_f$ 0.13±0.02); mobile phase toluene: ethyl acetate: diethyl-amine: glacial acetic acid (6.5: 5.0: 1.5: 0.5 v/v/v/v)**
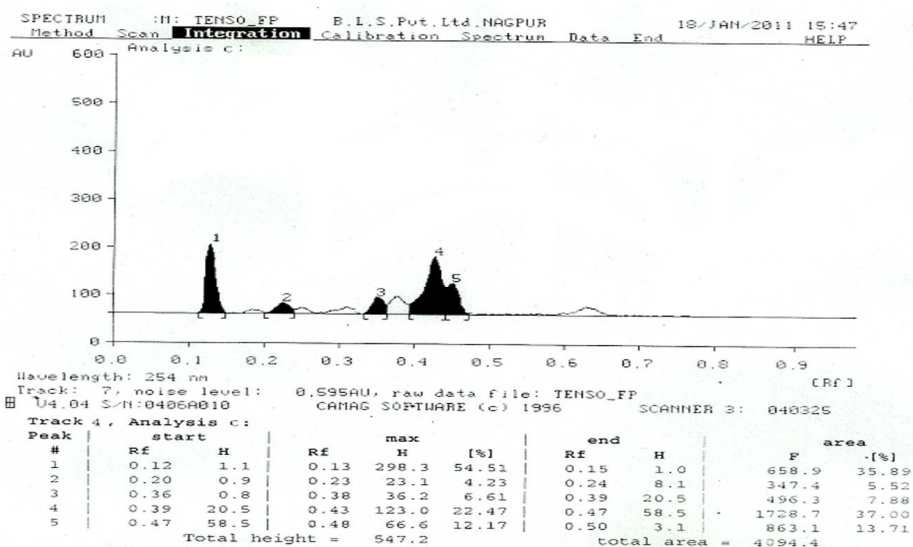


**Fig.6: Estimation of reserpine and arjunolic acid in Tensowert tablet (300μg/spot) peak 1-5 belongs to components in Tensowert tablet in which peak 1 is of arjunolic acid and peak 4 is of reserpine ($R_f$: 0.13±0.02 and 0.043±0.04); mobile phase; toluene: ethyl acetate: diethyl-amine: glacial acetic acid (6.5: 5.0: 1.5: 0.5 v/v/v/v)**

**Table 4: Estimation of reserpine in *Rauwolfia serpentina* extract**

| Component | Amount taken (μg/spot) | Peak area Mean ± S.D. | R.S.D. | Amount found (ng) | % Amount found |
|-----------|------------------------|------------------------|--------|-------------------|----------------|
| Reserpine Extract | 100 | 1320.3 ± 5.01 | 0.37 | 894.25 | 0.89 |

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**Table 5: Estimation of arjunolic acid in *Terminalia arjuna* extract**

| Component | Amount taken (µg/spot) | Peak area Mean ± S.D. | R.S.D. | Amount found (ng) | % Amount found |
|---|---|---|---|---|---|
| Arjuna Extract | 100 | 867.6 ± 5.25 | 0.60 | 1057.67 | 1.05 |

**Table 6: Estimation of reserpine and arjunolic acid in Tensowert tablet**

| Component | Amount taken (µg/spot) | Peak area Mean ± S.D. | R.S.D. | Amount Found (ng) | % Amount Found. |
|---|---|---|---|---|---|
| Reserpine | 300 | 1728.3 ± 5.70 | 0.33 | 1132.19 | 0.37 |
| Arjunolic acid | 300 | 658.3 ± 4.18 | 0.63 | 800.27 | 0.26 |

## CONCLUSION

There is significant difference between the $R_f$ values of the reserpine and arjunolic acid therefore this analytical method can be utilizes for the simultaneous estimation of these compounds. The proposed HPTLC method provide simple, accurate and reproducible quantitative analysis for simultaneous determination of reserpine and arjunolic acid. This method was validated as per ICH guideline. Statistical tests indicate that the proposed HPTLC method reduce the duration of analysis and appear to be equally suitable for routine determination of reserpine and arjunolic acid in herbal extract and formulation.

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