Research Article

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# Development and validation of a thin-layer chromatography-densitometric method for the quantitation of Isovanillin from *Hemidesmus indicus* (Linn.) R.Br., its extracts and marketed formulations

**Rajesh Kumar Verma*, Anubhuti Pasrija, Alok Sharma and Rahul Singh**
Dabur Research and Development Centre- Analytical Department,Dabur India Ltd. Company, Sahibabad, Ghaziabad, Uttar Pradesh, India

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## ABSTRACT

A selective, precise, and accurate high-performance thin-layer chromatographic (HPTLC) method has been proposed for the analysis of *Hemidesmus indicus* and its formulations for isovanillin content. *Hemidesmus indicus* is a twining shrub and a root of this taxon has been used in folk medicine as well as in ayurvedic and unani preparations. The method employed TLC aluminium plates precoated with silica gel 60 $F_{254}$ as the stationary phase. Linear ascending development with toluene: ethyl acetate: methanol: acetic acid 7.5: 1.5: 0.5:0.5 (v/v) as the mobile phase was performed at room temperature (25 ± 2ºC) in a twin-trough glass chamber saturated with mobile phase vapour. Compact bands (Rf 0.39 ± 0.02) were obtained for isovanillin in Crude plant materials, Herbal extracts and Formulations. Scanning was performed in absorbance mode at 230nm. Linear regression analysis of the calibration plots showed good linear relationship between peak area and peak height ($r^2$ = 0.9907 ±0.0002) in the concentration range 87-167 ppm. The method was validated for precision, recovery, robustness, specificity, detection and quantification limits. Recovery percentage in the sample found in between 84.14-86.73%. Quantification values of isovanillin in raw materials, extracts and marketed formulations were found to be 0.17%, 0.40% & 0.14% respectively. The developed technique is precise, specific and accurate for checking the purity of *H. indicus*.

***Keywords***: *Hemidesmus indicus*, Isovanillin, Formulations; HPTLC; Quantitation; Densitometric evaluation; Validation; Peak area.

## INTRODUCTION:

*H.indicus (Linn.)* R.Br. commonly known as Anantamula (Sanskrit) belongs to family Asclepiadeaceae. Extract of this plant is reported to possess anti-inflammatory, antipyretic, antioxidant (1) and anti-ulcerogenic properties (2). *H. indicus* extract is also found to inhibit lipid peroxidation and scavenge hydroxide radicals in vitro (3). It is effective against the diseases of blood, inflammation, respiratory disorders, syphilis, fever, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism (4). Isovanillin (3-Hydroxy-4-methoxy-benzaldehyde) present in the roots possesses anti-inflammatory, antipyretic and antioxidant activities (5). This compound was isolated from the plant previously but no HPTLC method for quantification of this compound in the plant has been reported so far. HPTLC finger print analysis has become the most potent tool for quality control of herbal medicines because of its simplicity and reliability. It can serve as a tool for identification, authentication and quality control of herbal drug (6). Standardization and quantification of isovanillin can serve as an important quality control tool for *Hemidesmus indicus*. The present paper deals with the development of a validated High Performance Thin Layer Chromatographic method for quantification of isovanillin in crude raw material, extracts and formulations of *H.indicus*. The developed HPTLC method is found to be sensitive, specific, precise and accurate for the identification and quantification of isovanillin in *H.indicus* in order to rationalize the medicinal benefit of the plant.

***Corresponding author.**
***Rajesh Kumar Verma***
***Drug Research and Development Centre- Analytical Department***
***Dabur India Ltd. Company,***
***Sahibabad, Ghaziabad,***
***Uttar Pradesh, India***

## 2. MATERIALS AND METHODS:

### Plant material:

The roots of *H.indicus* from three different regions of India named as Raw-1, Raw-2 and Raw-3 were procured and authenticated from Taxonomist. The marketed formulations of *H.indicus* were taken from Local market.

### Solvents and reagents:

All solvents and reagents used during chromatographic studies were of HPLC grade, supplied by Merck (Germany). Isovanillin was procured from Sigma-Aldrich, USA. The purity of standard was 99%.

### Instrument Used:

A Camag HPTLC system (Muttenz,Switzerland) equipped with a sample applicator Linomat V, twin trough plate development chamber, TLC Scanner and winCATS software.

**Chromatography Condition:**
HPTLC was performed on precoated silica gel 60 $F_{254}$ TLC plates (E. Merck, Germany) 20 cm ×10 cm. The samples were spotted in the form of bands of width 10 mm using a Camag 100 microliter sample syringe (Hamilton, Switzerland) with a Camag Linomat 5 applicator (Camag, Switzerland). The application parameters were identical for all the analysis performed and a constant application rate of 150 nL per second was used. Various combination of solvent systems were tried & finally the solvent system consisting of toluene: ethyl acetate: acetic acid: methanol (7.5:1.5:0.5:0.5,v/v) under laboratory conditions (25-30°C and 40-50% relative humidity) was finalized. Linear ascending development with mobile phase was carried out in a twin trough glass chamber (20 ×10 cm) previously saturated with mobile phase vapour for 30 minutes. After development up to 80 mm plates were air dried. Densitometric scanning was performed on Camag TLC scanner 3 in the absorbance mode at 230 nm. The slit dimension were 08 mm × 0.45 mm and scanning speed was 100 mm/s. Deuterium lamp was used as source of light emitting a continuous UV spectrum in the range 190-400nm.

**METHOD VALIDATION:**
The method was validated in raw material according to ICH guidelines (7), the method validation parameters checked were as follow:

**Precision:** Repeatability of sample application and measurement of peak area were carried out using six replicates of same spot of standard and test sample respectively and expressed in terms of % Relative Standard Deviation (%RSD) of peak area.

**Linearity:** A stock solution of isovanillin (417.2ppm) was prepared in methanol and dilution was done to obtained concentrations range of 87.61, 100.1, 112.6, 125.16, 137.6, 150.19 and 164.32 ppm respectively. The data of peak areas plotted against corresponding concentration were treated by least square regression analysis.

**Recovery:** The samples were spiked with extra 80,100 and 120% of standard isovanillin and the mixtures were analyzed by the proposed method in order to check the recovery of the isovanillin at different levels. Three determinations were performed at each level.

**Specificity:** It was ascertained by analyzing the standard drug and plant material. The spot for isovanillin was confirmed by comparing the $R_f$ values and spectra of the spot with that of standard. The peak purity of the isovanillin was assessed by comparing the spectra at three different levels, viz. peak start, peak apex and peak end position of the spot.

**Limit of Detection and Quantification:** In order to determine the detection and quantification limit, isovanillin concentration in the lower part of the linear range of the calibration curve was used.

**EXPERIMENTAL:**

**Preparation of raw materials:**
Accurately weighed 2gm of dried crude raw powder of *H. indicus* was transferred to round bottomed flask and refluxed for 30 minutes at room temperature (25 ± 5°C) with 25ml methanol. Filtered through Whatman filter paper (No. 41) and marc was again refluxed with 25ml methanol for 30 minutes. Filtrates were then combined and concentrated up to 25ml in rotary vacuum evaporator and the resulting solution was used as test solution.

**Preparation of methanolic extract of *H.indicus*:**
Methanolic extract of three raw materials named as Ext.-1, Ext.-2 & Ext.-3 were prepared by refluxing 200gm of crude raw material over water bath for 3-4 hours and left overnight. Next day filtered through Whatman filter paper (No.41) & the marc left out were refluxed again with methanol in the same manner for two days. All filtrates were combined and evaporated to dryness in rotary vacuum evaporator. About 0.2 gm of each extract was sonicated with 20 ml methanol & volume was made up to 25 ml with methanol and used as test solutions.

**Preparation of sample of Marketed formulations:**
Formulation like tablet and syrups used has been taken from commercial market. For formulation-1 (tablet), around 1gm of powdered tablet was sonicated with methanol for 10 minutes, filtered and used as test solution.

In case of syrup formulation-2 & 3, accurately weighed (25ml) syrup was dissolved in 25ml water and extracted with extraction media (chloroform: butanol: methanol, 25:3:1, v/v) three times, filtered through anhydrous sodium sulphate, filtrates were combined and concentrated up to 10ml in rotary evaporator and used as test solution.

**RESULTS AND DISCUSSION:**

**Development of Optimum Mobile Phase:**
Validation of analytical method is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results. It is an integral part of any good analytical practice. Various chromatographic techniques have been employed to judge the quality of analytical methods, among these High Performance Thin Layer Chromatographic technique have been extensively used in analytical chemistry for quality control, because of simplicity, high efficiency and speed (8). Chromatographic fingerprint is a rational option to meet the need for more effective and powerful quality assessment to Indian Traditional Medicine and Chinese traditional herbal medicine (9). The optimized chromatographic finger print is not only an alternative analytical tool for authentication, but also an approach to express the various patterns of chemical ingredients distributed in the herbal drugs. In order to develop and validate an efficient method for the analysis of isovanillin in crude raw materials, we explored different detection wavelengths, different solvent systems and the compositions of mobile phase. According to preliminary results, detection wavelength of 230 nm was finalized and the mobile phase toluene: ethyl acetate: acetic acid: methanol (7.5: 1.5: 0.5:0.5, v/v). The mobile phase Toluene: Ethyl acetate: Acetic acid: Methanol gave good resolution, dense,

compact and well separated spots of isovanillin as well as well defined peak at $R_f$ =0.39 for isovanillin. This is the first such validated analytical method for the quantification of isovanllin in *H. indicus.*

**VALIDATION OF THE METHOD:**

**System precision:** Repeatability of sample application and measurement of peak areas were carried out using six replicates of same spot of standard. Data shown in Table 1 indicate an acceptable level of precision for analytical system. (Acceptance criteria: %RSD should not be more than 2.0 %). Standard peak chromatogram is shown in Fig 1.

**Table 1. System precision data for the determination of isovanillin**

| Area | | | | | | Mean area | SD | %RSD |
|---|---|---|---|---|---|---|---|---|
| 9220.9 | 8998.2 | 8979.3 | 9079.5 | 8949.1 | 8990.8 | 9036.3 | 100.32 | 1.11 |

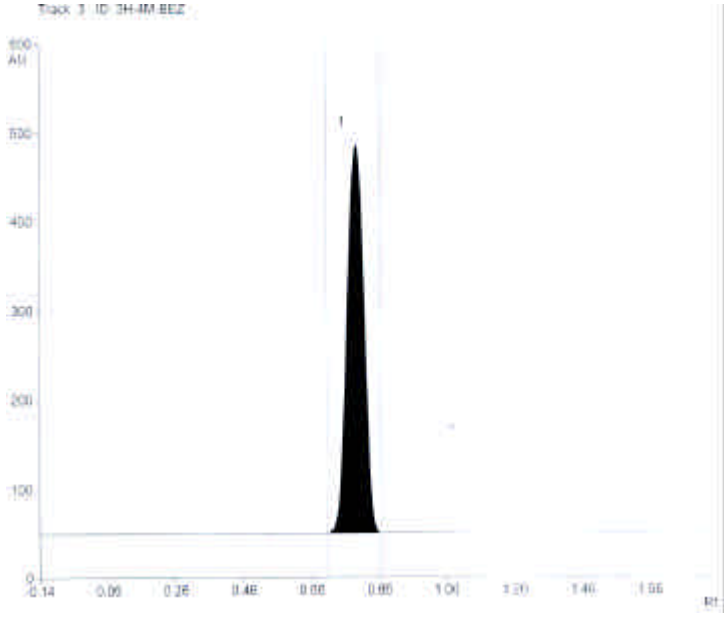

**Figure 1. HPTLC Chromatogram of standard Isovanillin**

**Method precision:** %RSD for the repeatability of sample application of a single batch of raw material (Raw-2) was 9.2%. The % RSD values indicate that the method has an acceptable level of precision as shown in Table 2. Sample chromatogram shown in Fig.2. (Acceptance criteria: %RSD should not be more than 10.0 %).

**Table 2. Method precision data for the determination of isovanillin**

| Concentration of Isovanillin (%w/w) | | | | | | Mean area | SD | %RSD |
|---|---|---|---|---|---|---|---|---|
| 0.144 | 0.132 | 0.163 | 0.172 | 0.154 | 0.154 | 0.153 | 0.014 | 9.2 |

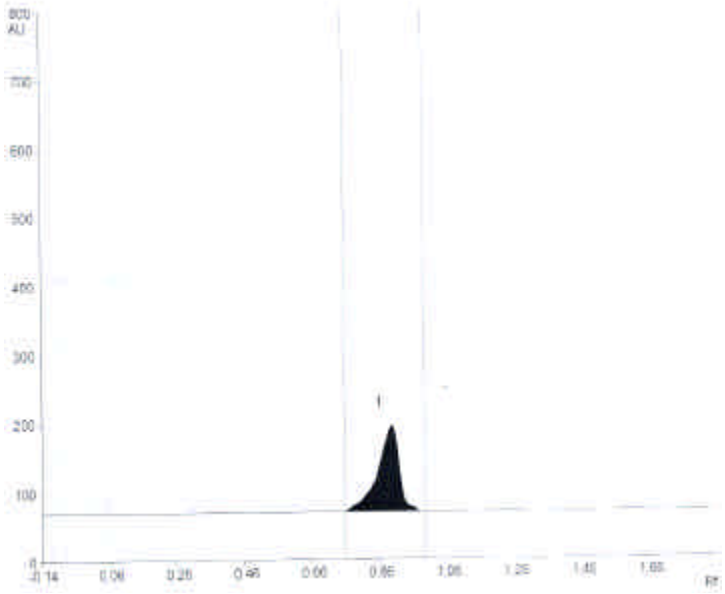

**Figure 2 HPTLC Chromatogram of Isovanillin in Raw material**

**Calibration curve and linearity:** A good linear relationship (correlation coefficient $r^2$ = 0.9907) for isovanillin was obtained with the developed method over the range as shown in Table 3 and represented graphically in Fig. 3 which indicates that the response is linear over the specified range.

**Table 3. Calibration parameters for isovanillin**

| Sr.No. | Conc. of Standard isovanillin | Peak area |
|---|---|---|
| 1 | 87.61 | 4796.5 |
| 2 | 100.1 | 5317.2 |
| 3 | 112.6 | 5931.5 |
| 4 | 125.16 | 6213.4 |
| 5 | 137.6 | 6686.9 |
| 6 | 159.19 | 6989 |
| 7 | 164.3 | 7703.3 |

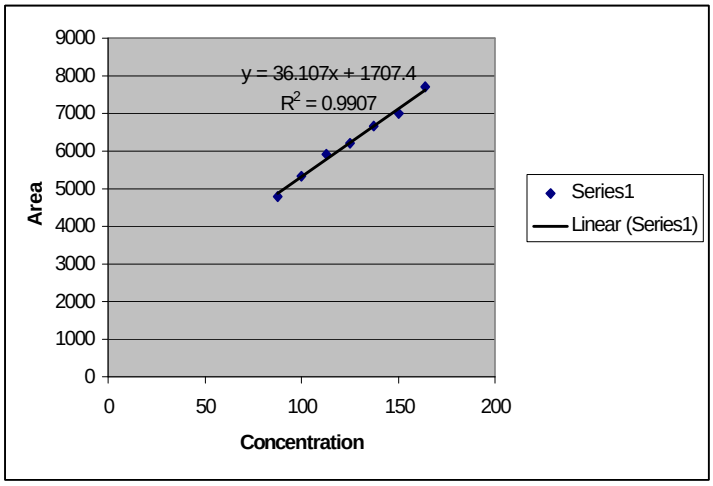


**Figure 3. Linearity plot of Isovanillin**

**Specificity:** No interference from placebo was observed at the resolution factor of isovanillin. The peak purity plots also indicate that the peak of isovanillin is pure and has no co-eluting peaks. Therefore, it was concluded that the method is specific.

**Accuracy:** The proposed method when used for quantification of isovanillin from raw material after spiking with standard show good recovery in between 84.3 % to 86.7%. (Table 4)

**Table 4. Recovery of isovanillin in *Hemidesmus indicus* sample**

| Recovery level | Isovanillin Amount added (ppm) | Amount recovered (ppm) | % Recovery | Average Recovery |
|---|---|---|---|---|
| 80 % Rec. A | 193.40 | 163.09 | 84.3 | |
| 80 % Rec. B | 193.40 | 162.45 | 83.99 | |
| 80 % Rec. C | 193.40 | 162.74 | 84.14 | 84.14% |
| 100 % Rec. A | 211.67 | 183.15 | 86.52 | |
| 100 % Rec. B | 211.67 | 183.98 | 86.91 | |
| 100 % Rec. C | 211.67 | 183.67 | 86.77 | 86.64% |
| 120 % Rec. A | 230.37 | 196.85 | 85.44 | |
| 120 % Rec. B | 230.37 | 197.77 | 85.84 | |
| 120 % Rec. C | 230.37 | 197.32 | 85.65 | 85.65% |

**LOD and LOQ:** The sensitivity of the method was determined in terms of Limit of Quantification (LOQ), linearity range and correlation coefficient. After densitometric analysis of isovanillin at 230nm, the lowest amount of isovanillin, which could be detected was found to be 11.69 ppm and the lowest amount of isovanillin which could be quantified was found to be 14.04 ppm.

**Estimation of isovanillin in raw materials, extracts & its formulations:**

An little variation was observed in the content of isovanillin in the raw materials from three different regions & their extracts as shown in Table 5. HPTLC chromatogram of extracts is shown in Fig. 4. In case of formulations, quantification was quite difficult due to the presence of trace amount of isovanillin in formulations. Only tablet formulation showed the peak for isovanillin whereas detection was not possible in case of other two formulations. HPTLC chromatogram of tablet formulation is shown in Fig. 5.

**Table 5. Quantification values of Isovanillin in raw materials, extracts and marketed formulations of *Hemidesmus indicus***

| S.No. | Sample | Conc.(%w/w) |
|---|---|---|
| 1 | Raw-1 | 0.168 |
| 2 | Raw-2 | 0.153 |
| 3 | Raw-3 | 0.142 |
| 4 | Extract 1 | 0.271 |
| 5 | Extract 2 | 0.395 |
| 6 | Extract 3 | 0.308 |
| 7 | Formulation 1 | 0.136 |
| 8 | Formulation 2 | - |
| 9 | Formulation 3 | - |

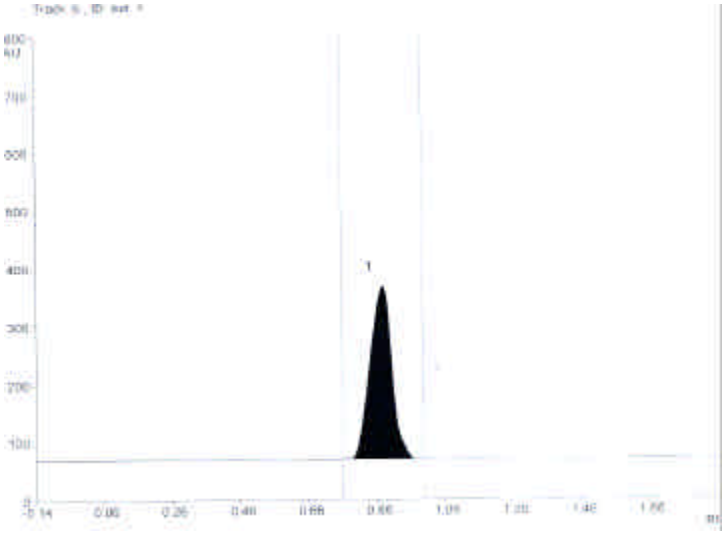

**Figure 4. HPTLC Chromatogram of Isovanillin in Extract**

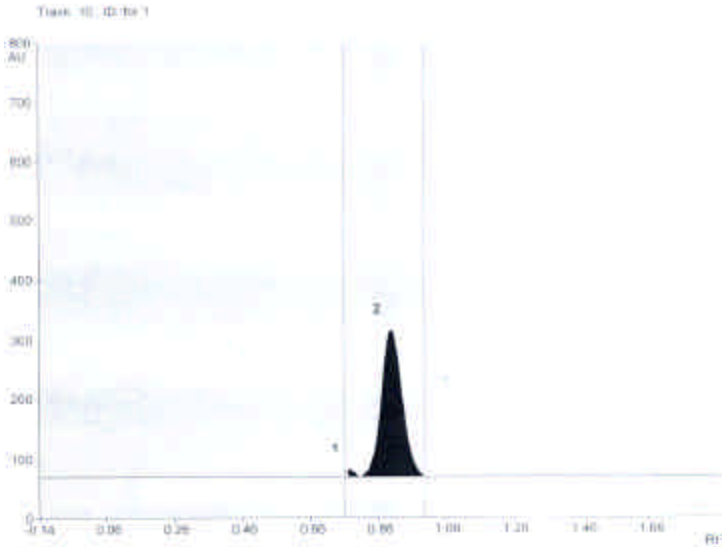

**Figure 5. HPTLC Chromatogram of Isovanillin in Formulation**

**CONCLUSION:**

This developed and validated HPTLC method for determination of isovanillin is simple, reliable, precise, and specific and show good recovery. Since the resolution of isovanillin was good, the method can be used for both qualitative and quantitative analysis of isovanillin without interference of other constituents. Statistical analysis proves the method is reproducible and selective for the analysis of isovanillin.

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