QUANTITATIVE ESTIMATION OF PARA HYDROXY BENZOIC ACID AND PARA HYDROXYBENZALDEHYDE IN VANILLA PLANIFOLIA PODS BY HPTLC

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ABSTRACT
This study presents the report of quantitative estimation of para hydroxy benzoic acid and para hydroxybenzaldehyde in Vanilla planifolia using HPTLC. Silica gel aluminum plate 60 F254 (5 cm ×10 cm) with 250 μm thickness were used for separation. Good separation was achieved by using mobile phase n-hexane: ethyl acetate (7: 3 v/v). Determination and quantitation were performed by densitometric scanning at 266nm. The amount of para hydroxyl benzoic acid present was found to be 27.28µg/ml and amount of para hydroxyl benzaldehyde present was found to be 47.01µg/ml. The HPTLC method can be used for quantification of para hydroxy benzoic acid and para hydroxyl benzaldehyde in crudedrugs, herbal drugs and formulation composed of several components.

Key words: Para hydroxy benzoic acid, para hydroxybenzaldehyde, Vanilla planifolia, HPTLC.

INTRODUCTION
HPTLC is a powerful modern analytical tool which is suitable for qualitative and quantitative analytical tasks. It works as a complementary method to HPLC. HPTLC produces visible chromatograms giving complex information about the entire sample at a glance. Similarities and differences can be identified with the help of the image comparison. Several chromatograms can be compared directly. In addition to the visible chromatograms, analog peak data are also available from the chromatogram. They can be evaluated either by the
image based software or by scanning densitometry with TLC Scanner[1]. Development of various analytical techniques for the analysis of phytoconstituents is an important step in the standardization of herbal drugs. HPTLC is an inexpensive method for qualitative and quantitative analysis of plant samples. *Vanilla planifolia* belongs to the family Orchidaceae. It is a thick, tropical, leafy, ever green climbing orchid, with fleshy leaves. It adheres to the trees by means of aerial roots. There are clusters of trumpet shaped flowers with three sepals which are 4-7 cm long [2].

The constituents reported to be present in this plant are vanillic acid, anisaldehyde, hydroxy benzoic acid, anisylalcohol, caproic acid, vitispiranes, eugenol, phenols, phenol ether, carbonyl compounds, acids, esters, ether, 25% carbohydrates, 15% fat, and B complex[3].

p-hydroxybenzoic acid is a monohydroxybenzoic acid, a phenolic derivative of benzoic acid. It is present in many plants like *Citrus paradisi, Olea europaea, esculentus, Daucus carota, Elaeis guineensis,* etc[4]. It has been reported to possess antifungal, antimutagenic, antisickling, estrogenic and antimicrobial activities[5, 6]. It has also been reported to possess preservative action[7]. para-Hydroxybenzaldehyde is one of the three isomers of hydroxybenzaldehyde. It has been reported to possess, antioxidant activity, anti-inflammatory, positive modulation of GABAergic neuromodulation, Inhibition of GABA shunt enzymes' activity[8-10].

**METHODOLOGY**

**Apparatus used for HPTLC**

Sample syringe (Hamilton), Sample applicator (CAMAG Linomat 5), Development chamber (twin trough glass Chamber, CAMAG), Densitometric scanner (CAMAG thin layer chromatography scanner).

**Chemicals**

Methanol, ethylacetate, hexane, chloroform, para hydroxy benzoic acid and para hydroxybenzaldehyde (S.D Fine Chemicals Ltd. Mumbai)

**Extraction of plant material**

The dried pods of *Vanilla planifolia* were subjected to repeated extraction. This process was carried out three times using methanol as the solvent. The extracts were combined, filtered,
and powdered to obtain a uniform particle size. This uniform homogenous powdered extract was heat sterilized and packed.

**Preparation of plant extract sample**

The plant extract (50mg) was dissolved in 10ml of methanol. This solution was applied on the chromatographic plates as bands

**TLC of phytoconstituents**

Para hydroxy benzoic acid was acidified and extracted with diethyl ether. Separation was done on Silica gel G. The developing solvent used was toluene: methanol: acetic acid (90: 16: 8). It was observed as a black spot at a wavelength of 254 nm. The Rf value of the parahydroxy benzoic acid standard was found to match the Rf value of the plant extract[11].

P- hydroxybenzaldehyde was estimated using Thin layer chromatography. The stationary phase used was silica gel G. The eluting solvent used was petroleum spirit: ethoxy ethane.

The detecting reagent used was 2,4 DNPH. The spots were yellow – orange colour. The Rf value of the plant extract was 0.35 which matched the Rf value of the para hydroxybenzaldehyde standard [12].

**Standard preparation of para hydroxy benzoic acid**

The para hydroxy benzoic acid standard (10mg) was dissolved in 10ml of methanol. The resulting solution had a concentration of 1000 µg/ml. From the above solution, 1ml was pipetted out and diluted to 10ml. The resulting solution has a concentration of 100 µg/ml (100 ng/µl). This solution was spotted as bands of volume 1-5 µl, thus each band had a concentration of 100-500ng. Thus the calibration curve for parahydroxy benzoic acid was obtained.

**Standard preparation of para hydroxybenzaldehyde**

The para hydroxybenzaldehyde standard (10mg) was dissolved in 10ml of methanol. The resulting solution had a concentration of 1000 µg/ml. Then pipetted out 1ml from the above solution and diluted it to 10 ml. The resulting solution has a concentration of 100 µg/ml (100ng/µl). This solution was spotted as bands of volume 1-6 µl, thus each band had a concentration of 100-600ng. Thus the calibration curve for para hydroxybenzaldehyde was obtained.
Solvent system
The solvent system which gave good resolution for para hydroxy benzoic acid and para hydroxybenzaldehyde was n-hexane and ethyl acetate in a ratio of 7:3 v/v

Sample application
The samples were spotted (10 µL) in the form of bands, having a width of 6 mm, with space between bands of 8 mm, using a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) on precoated silica gel aluminium plate 60 F254 (5 cm ×10 cm) with a thickness of 250 µm (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). The slit dimensions were 5 mm × 0.45 mm. A scanning speed of 20 mm/sec was employed

Development of chromatogram
Development of the chromatogram was done by linear ascending development method. It was carried out in a 10 cm×10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) using n-hexane: ethyl acetate (7: 3 v/v) as mobile phase. The optimized chamber saturation time for mobile phase was 20 min. The length of chromatogram run was 8 cm and development time was approximately 15 min. TLC plates were dried in a current of air with the help of a hot air drier

Detection of spots
Densitometric scanning was performed on a CAMAG thin layer chromatography scanner at a Wave length of 266 nm. The scanner was operated by WINCATS software version 1.4.2

RESULTS
The samples were spotted (10 µL) in the form of bands of width of 6 mm with space between bands of 8 mm, with a 100 µL sample syringe on precoated silica gel aluminium plate 60 F254 (5 cm ×10 cm) with 250 µm thickness using a CAMAG Linomat 5 sample applicator.

The slit dimensions 5 mm × 0.45 mm and scanning speed of 20 mm/sec was employed The Rf value of para hydroxy benzoic acid was found to be 0.17 and para hydroxyl benzaldehyde was 0.42. Rf values of all tracks are as shown as a 3Ddiagram in fig 1 along with their areas are mentioned in table 1. Chromatograph showing para hydroxyl benzoic acid and para hydroxybenzaldehyde in standard is as shown in fig 2. While fig 3 and 4 represent the insitu overlay of the para hydroxy benzoic acid and para hydroxybenzaldehyde. Fig 5 and 6 depicts
the calibration curves. Amount of para hydroxyl benzoic acid present was found to be 27.28µg/ml, while amount of para hydroxyl benzaldehyde present was found to be 47.01135µg/ml.

![D chromatograph showing para hydroxy benzoic acid and para hydroxy benzaldehyde](image)

**Figure 1: D chromatograph showing para hydroxy benzoic acid (Rf -0.17) and para hydroxy benzaldehyde (Rf -0.42)**

**Table 1: Peak areas of p hydroxy benzoic acid and para hydroxy benzaldehyde**

<table>
<thead>
<tr>
<th>Concentration (ng/band)</th>
<th>Area (p-hydroxy benzoic acid) Rf 0.17</th>
<th>Area (p-hydroxybenzaldehyde) Rf 0.42</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>697</td>
<td>17181.1</td>
</tr>
<tr>
<td>200</td>
<td>1586</td>
<td>23457.3</td>
</tr>
<tr>
<td>300</td>
<td>2561.4</td>
<td>29713</td>
</tr>
<tr>
<td>400</td>
<td>3669.2</td>
<td>34737.2</td>
</tr>
<tr>
<td>500</td>
<td>4608.7</td>
<td>39406.2</td>
</tr>
<tr>
<td>600</td>
<td>5789.5</td>
<td>44587.8</td>
</tr>
<tr>
<td>Track 1 (5 µl)</td>
<td>1001.5</td>
<td>25348.1</td>
</tr>
<tr>
<td>Track 2 (10 µl)</td>
<td>2318.3</td>
<td>37887.6</td>
</tr>
</tbody>
</table>
Fig 2: Chromatograph showing para hydroxy benzoic acid and para hydroxybenzaldehyde in standard

Track 1 – 6: Standard para hydroxy benzoic acid (Rf -0.17) and para hydroxy benzaldehyde (Rf -0.42) in the concentration range 100-600 ng/band

Track 7 – 8: Sample solution (5 µl and 10 µl respectively)

Track 9 – 10: Methanol blank

Fig 3: In situ overlaid spectrum of para hydroxy benzoic acid in standard and in sample at Rf -0.17
Fig 4: In situ overlaid spectrum of para hydroxybenzaldehyde in standard and in sample at Rf -0.42

Fig 5: In situ overlaid spectrum of spot in sample at Rf -0.53

Fig 6: Chromatographic plate at 254nm
A1 – Para hydroxybenzaldehyde (10µl)
A2 – Para hydroxybenzaldehyde (5µl)
B1 – Para hydroxy benzoic acid (10µl)
B2 – Para hydroxy benzoic acid (5µl)
C1 & C2 – Standard solution containing para hydroxy benzoic acid & para hydroxybenzaldehyde. (6µl)

Table 2: Concentrations of para hydroxyl benzoic acid and para hydroxybenzaldehyde in track 1 and track 2

<table>
<thead>
<tr>
<th>Track</th>
<th>Concentration</th>
<th>Track 1</th>
<th>Track 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>138.811 ng/band</td>
<td>236.373 ng/band</td>
</tr>
<tr>
<td>Track 1</td>
<td></td>
<td>268.137 ng/band</td>
<td>467.481 ng/band</td>
</tr>
</tbody>
</table>

Fig 7: Calibration curve for p hydroxy benzoic acid

Fig 8: Calibration curve for p hydroxybenzaldehyde
CONCLUSION
HPTLC is an important method for development of chromatographic fingerprints to determine the phytoconstituents of medicinal plants. The separation and resolution are much more reliable and reproducible than TLC. It has the main advantage of in situ quantitative measurement by scanning densitometry. The results obtained from qualitative evaluation of HPTLC fingerprint images can be utilized for the identification and quality control of the drug. Hence HPTLC analysis of these compounds from the pods of Vanilla planifolia can be used as a reference for the identification of extracts in which these are constituents.

REFERENCES
