Development and Validation of Stability Indicating High Performance Thin Layer Chromatography Method for Analysis of Bergapten


*JSPM’s Rajarshi Shahi College of Pharmacy and Research, Talewadi, Pune-411033, Maharashtra, India.

Abstract:
Bergapten, a phytoconstituent has many therapeutic activities such as antimicrobial, anti-inflammatory, anticancer, antioxidant, anticonvulsant, and osteoporosis activities. Owing to increased demand of standardization of herbal drugs and their formulations, it is essential to know degradation pathways for bergapten, which would give direction about its formulation development, packaging and storage conditions. A simple, precise, accurate and rapid stability-indicating High-Performance Thin Layer Chromatography (HPTLC) method was developed for bergapten. For development of chromatograms, toluene: dichloromethane: ethyl acetate (7:2:1 v/v/v) was used as mobile phase. The densitometric scanning was performed at 318 nm. The method was found linear over from 25 to 400 ng/band with correlation coefficient 0.998. The developed HPTLC method was validated as per ICH guidelines. Validated HPTLC method was used to reveal the degradation products of bergapten after it was subjected to acid and alkali induced degradation, oxidative, thermal and photolytic degradation. Degradation products from each of the above degradation pathways were revealed. The developed method was stability indicating. The proposed method would be able to selectively analyze bergapten and its degradation products in drug substance and its formulation.

Keywords: Bergapten, forced degradation, HPTLC, ICH, stability indicating.

1. INTRODUCTION

Bergapten is also known as 5-methoxypsoralen belongs to the chemical class of furanocoumarins. Bergapten is present in various plant parts such as root, stem bark, fruits, and leaves. It finds in plant species of family Moraceae, Umbelliferae, Apioaceae, Rutaceae [1,2]. It has been used as remedy in various disorders and diseases. It has been used to treat psoriasis, vitiligo and atopic inflammation [3]. It has been found effective in controlling liver cancer by changing the multiple lipogenic pathways and has been used as an anticancer agent [4,5]. It has been used as an anti-inflammatory agent with a dose of 5, 10 and 20 µg/ml which is effective in inhibiting the number of macrophages and neutrophils at the site of injury, also it is effective in preventing lipopolysaccharide-induced inflammation [6,7]. Bergapten is one of the main constituents of bergamot oil which exhibited DPPH scavenging activity at a concentration of 63.38 µg/ml [8]. Bergapten isolated from bark extract of Ficus religiosa has exhibited good antimicrobial activity [9]. Bergapten has showed anticonvulsant activity from fruits of Heracleum crenatifolium [10].

In the current era, there is a continuous increase in demand of herbal formulations for therapeutic purpose to subside the adverse effect of synthetic drugs. Regulatory authorities are posing to standardize the herbal drugs and their formulations. Therefor it is essential to standardized herbal drugs to comply the regulations. From literature survey it was revealed that high performance liquid chromatography (HPLC), HPTLC, High-speed counter-current chromatography (HSCCC) analytical methods were reported for analysis of bergapten [11-15]. But no any stability indicating HPTLC method was report so far for the analysis of bergapten. The objective of the present work was to develop and validate the stability indicating HPTLC method for bergapten. Stability indicating analytical method (SIAM) is a validated analytical method that accurately and precisely separates and analyze the drug from its possible interferences like degradation products, excipients, or impurities in drug product [16]. SIAM provides the information of degradants, degradation pathways of drug substance and drug products. It helps in determining shelf life of active pharmaceutical ingredient (API) and its formulation. This information would be helpful for determining packaging and storage conditions of drug substance and drug products. After development of stability indicating method, one can proceed for structure elucidation of degradants by suitable advanced methods such as LC-MS, GC-MS [17-19].

2. EXPERIMENTAL

2.1. Materials and methods
Bergapten (99.7% w/w) was procured from Maha Gauri Natural Products, India. All chemicals and reagents were of analytical grade and procured from Merck, India.

2.2. HPTLC instrument
Camag Hamilton (100 µL) syringe was used for sample application on precoated silica plates 60F 254, Merck. Camag Linomat V, an automatic sample applicator attached with nitrogen gas unit was used for application of sample on TLC plates. The sample application was done in the form of bands of length 6 mm. Camag twin trough chamber (20 cm × 20 cm) was used for washing the TLC plates. The chromatographic development was done in a linear ascending manner in Camag twin trough chamber (20 cm × 10 cm). Camag TLC scanner 3 (Muttenz, Switzerland) was used for densitometric analysis.

Other operating parameters used were, slit dimensions: 5.00×0.45 mm, migration distance: 14 mm, distance between bands: 10.0 mm, sample application position: 8.0
mm, solvent front position: 80.0 mm, scanning speed: 20
mm/s.

2.3. Preparation of solutions
2.3.1. Preparation of standard solution
A standard stock solution of bergapten was prepared by
dissolving accurately weighed amount of drug in
methanol to get the concentration of 500 µg/ml.
2.3.2. Preparation of standard working solution
Standard stock solution was diluted with sufficient
methanol to get the concentration of 50 µg/ml.
2.3.3. Preparation of sample stock solution
Sample equivalent to 5 mg of bergapten was dissolved in
sufficient methanol to produce a concentration of 500
µg/ml. The resulting solution was sonicated for 15 min
and filtered through the Whatman filter paper.

2.4. Characterization of bergapten
Characterization of the drug was done by determination of
melting point using capillary method and recording FTIR
spectra of drug (Fig. 1.), (Table 1). The functional groups
were reported. An absorbance maximum of bergapten was
determined by scanning TLC plate from 200-400 nm (Fig.
2). The solubility of bergapten was determined in different
solvents for selection of mobile phase.

2.5. Analysis of bergapten
2.5.1. Optimization of mobile phase
Optimization of mobile phase was carried out using different mobile phase compositions for chromatographic
development (section 4.2). The chromatograms were
observed for peak shape and R_s value. The suitable mobile
phase composition was selected for further work (Fig. 3).
2.5.2. Linearity study of bergapten
Aliquots of standard working solution (50 µg/ml), were
spotted (concentration of 25, 50, 100, 150, 200, 250, 300,
350, 400 ng/band) on pre-coated TLC plates. The
optimized mobile phase was allowed to saturate for 10 min
and the plates were developed, vacuum dried and scanned.
The calibration curve was obtained by plotting peak area
versus concentration (Fig. 4,5), (Table 2). The regression
coefficient and regression equation were obtained.
2.5.3. Analysis of bergapten in the formulation
Sample solution of 150 ng/band was applied on the TLC
plate in the form of bands in triplicate. The chromatograms were developed and scanned at 318 nm.
Drug content was analyzed (Table 3).

2.6. Validation of method
The developed HPTLC method was validated as per ICH
guidelines [20].
2.6.1. Specificity
Specificity was ascertained by applying the standard drug
solution, sample solution, diluent and mobile phase on
TLC plate, the plate was developed and scanned to verify
chances of interference if any (Fig. 6).
2.6.2. Precision
System precision was ascertained by application of
standard solution of bergapten, 150 ng/band, six times on
TLC plates (Table 4). The intra-day and inter-day
precision were performed by applying, 150, 200, 250
ng/band in triplicate (Fig. 7), (Table 5).

2.6.3. Recovery
The recovery of bergapten was studied at three levels. The
pre-analyzed sample solution was spiked at 80, 100, 120% levels with standard bergapten; these solutions were
analyzed in triplicates (Table 6).
2.6.4. Robustness of method
It was established by modifying mobile phase saturation
time (5 min and 15 min) and mobile phase composition
toluene: dichloromethane: ethyl acetate (8:1:1 and 6:3:1
v/v/v) and observing the retardation factor (Table 7).
2.6.5. Limit of detection (LOD) and limit of quantification
(LOQ)
LOD and LOQ were determined by using the standard
deviation of the y-intercept of the regression line as per
ICH guideline method (Table 8).

3. Forced degradation study
3.1. Acid hydrolysis degradation
Accurately weighed quantity of bergapten was dissolved in
sufficient methanolic HCl, (1 M). This solution was
refluxed at 80 °C for 1 h in dark to avoid interference of
light. The resulting solution was diluted up to 10 ml
methanol and applied as 150 ng/band, the chromatogram was developed and scanned (Fig. 8). The chromatograms
chromatogram was for degradation products and percent
of drug degraded.
3.2. Base hydrolysis degradation
Accurately weighed quantity of bergapten was dissolved in
sufficient methanolic NaOH 0.1 M. This solution was
refluxed at 80 °C for 2 h in dark to avoid interference of
light. The resulting solution was diluted up to 10 ml
methanol and applied as 150 ng/band on the plate. The
chromatogram was developed and scanned (Fig. 9). The chromatogram was observed for degradation products and percent of drug degraded.

3.3. Hydrogen peroxide degradation
Accurately weighed quantity of bergapten was dissolved in
sufficient 3% methanolic hydrogen peroxide. This
solution was refluxed at 80 °C for 1 h in dark to avoid
interference of light. The resulting solution was diluted up
to 10 ml methanol and applied as 150 ng/band, on the
plate. The chromatogram was developed and scanned (Fig.
10). The chromatogram was observed for degradation products and percent of drug degraded.

3.4. Thermal degradation
Accurately weighed quantity of bergapten was kept in the
oven at 100 °C for 1 h. It was then dissolved in sufficient
methanol and applied as 150 ng/band on the TLC plate.
The chromatogram was developed and scanned (Fig. 11).
The chromatogram was observed for degradation products and percent of drug degraded.

3.5. Photochemical degradation
The 5 mg of bergapten was spread on a Petri plate and
kept in a UV chamber at 254 nm for 24 h. It was then
dissolved in sufficient methanol and applied as 150
ng/band, the chromatogram was developed and scanned (Fig. 12). The chromatogram was observed for
degradation products and percent of drug degraded.
The results of above study were summarized in Table 9.
4. RESULTS AND DISCUSSION

4.1. Characterization of bergapten

4.1.1. Melting point of bergapten

The melting point of bergapten was found in the range of 188 °C-190 °C.

4.1.2. IR spectrometry

![Infrared spectrum of bergapten](image1.png)

**Table 1. Spectral characterization.**

<table>
<thead>
<tr>
<th>Wavenumber cm⁻¹</th>
<th>Functional groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>3071</td>
<td>Ar. C-H</td>
</tr>
<tr>
<td>2880</td>
<td>C-H of the methoxy group</td>
</tr>
<tr>
<td>1651</td>
<td>C=O group</td>
</tr>
<tr>
<td>1537</td>
<td>Ar. C=C</td>
</tr>
<tr>
<td>1232</td>
<td>C-O-C ether</td>
</tr>
</tbody>
</table>

![Absorption maxima of bergapten](image2.png)

4.1.3. Absorption maxima of bergapten

Absorption maxima of bergapten was found 318 nm (Fig. 2).

4.1.4. Solubility of bergapten

Bergapten is freely soluble in methanol, ethanol and toluene, and slightly soluble in chloroform, benzene and glacial acetic acid.

4.2. Analysis of bergapten

4.2.1. Optimization of mobile phase

The different ratios of solvents toluene, dichloromethane and ethyl acetate 7:3:0.1 v/v/v, 8:2:0.1 v/v/v and 7:2:1 v/v/v were tried for developing the chromatograms for optimizing the mobile phase.

![Chromatogram of bergapten](image3.png)

Fig. 3. Chromatogram of bergapten, mobile phase-toluene: dichloromethane: ethyl acetate (7:2:1 v/v/v) and $R_f$ 0.56.

A sharp peak of bergapten with 0.56 $R_f$ was obtained with mobile phase toluene: dichloromethane: ethyl acetate composition 7:2:1 v/v/v which was selected for further work (Fig. 3).

4.2.2. Determination of linearity and analytical range of bergapten

![Calibration curve of bergapten](image4.png)

Fig. 4. Calibration curve of bergapten, analytical range-25-400 ng/band, at 318 nm wavelength.

![Three dimensional graph of bergapten](image5.png)

Fig. 5. Three dimensional graph of bergapten for linearity study.
The method was found linear over the concentration range 25-400 ng, with correlation coefficient 0.998.

4.2.3. Analysis of bergapten in the formulation

Table 3. Analysis of bergapten in the formulation (*n=3).

<table>
<thead>
<tr>
<th>Conc. (ng/band)</th>
<th>Mean* conc.(ng)</th>
<th>% found ±SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>149.3</td>
<td>99.57% ±0.277555</td>
<td>0.278732</td>
</tr>
</tbody>
</table>

Using proposed HPTLC method, the formulation was analyzed in triplicate with SD ± 0.277555 and %RSD 0.278732 which were within the acceptance limit.

4.3. Validation of method

4.3.1. Specificity

Specificity study revealed no interferences of mobile phase, diluent and excipient in the analysis of bergapten by developed HPTLC method. (Fig. 6).

4.3.2. System precision

Fig. 7. Three dimensional graph for system precision.

Table 4. System precision (*n=6).

<table>
<thead>
<tr>
<th>Conc.(ng/band)</th>
<th>Mean area</th>
<th>SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>4555.24</td>
<td>10.00</td>
<td>0.21</td>
</tr>
</tbody>
</table>

The method was found repeatable with %RSD 0.21, which was well within the acceptance limit.

4.3.3. Method Precision

The method was found precise with standard deviation and relative standard deviation values well within the acceptance limit.

4.3.4. Recovery study

Method exhibited good recovery of bergapten with % recovery from 99.78-99.85%.

Table 5. Recovery studies (*n=3).

<table>
<thead>
<tr>
<th>Levels of Standard addition (%)</th>
<th>Theoretical content (ng)</th>
<th>Mean Recovery (%)</th>
<th>SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>270</td>
<td>99.78</td>
<td>0.75</td>
<td>0.28</td>
</tr>
<tr>
<td>100</td>
<td>300</td>
<td>99.85</td>
<td>0.92</td>
<td>0.31</td>
</tr>
<tr>
<td>120</td>
<td>330</td>
<td>99.79</td>
<td>0.64</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Fig. 6. Three dimensional graph for specificity study.
4.3.5. Robustness of the method

Table 7. Robustness of the method (*n = 3).

<table>
<thead>
<tr>
<th>Conc.(ng/band)</th>
<th>Parameters</th>
<th>Parameters</th>
<th>Ri</th>
</tr>
</thead>
<tbody>
<tr>
<td>150</td>
<td>Optimized mobile phase</td>
<td>Toluene: dichloromethane: ethyl acetate (7:2:1 v/v/v)</td>
<td>0.56</td>
</tr>
<tr>
<td>150</td>
<td>Mobile phase composition</td>
<td>Toluene: dichloromethane: ethyl acetate (8:1:1 v/v/v)</td>
<td>0.53</td>
</tr>
<tr>
<td>150</td>
<td>Mobile phase composition</td>
<td>Toluene: dichloromethane: ethyl acetate (6:3:1 v/v/v)</td>
<td>0.54</td>
</tr>
<tr>
<td>150</td>
<td>Mobile phase saturation time</td>
<td>5 minutes</td>
<td>0.57</td>
</tr>
<tr>
<td>150</td>
<td>Mobile phase saturation time</td>
<td>15 minutes</td>
<td>0.53</td>
</tr>
</tbody>
</table>

The method was not significantly affected by slight change in the mobile phase composition and mobile phase saturation time. Therefore, the method was found to be robust.

4.3.6. Limit of detection (LOD) and limit of quantification (LOQ)

Table 8. Limit of detection and limit of quantification for bergapten.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>10.17 ng</td>
</tr>
<tr>
<td>LOQ</td>
<td>30.82 ng</td>
</tr>
</tbody>
</table>

The values of LOD and LOQ ascertained that the method was quite sensitive.

4.4. Forced degradation study

Fig. 8. Chromatogram for acid induced degradation of bergapten.

Fig. 9. Chromatogram for alkali induced degradation of bergapten.

Fig. 10. Chromatogram for hydrogen peroxide induced degradation of bergapten.

Fig. 11. Chromatogram for dry heat induced degradation of bergapten.

Table 9. Forced degradation of bergapten.
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Forced degradation condition</th>
<th>Number of degradation products (Rf value)</th>
<th>Figure</th>
<th>% Drug recovered</th>
<th>% Drug degraded</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1N HCL 80ºC 1h</td>
<td>4 (0.04, 0.83, 0.90, 0.96)</td>
<td>Fig.8</td>
<td>91.16</td>
<td>8.83</td>
</tr>
<tr>
<td>2</td>
<td>0.1N NaOH 80ºC 2h</td>
<td>3 (0.03, 0.12, 0.89)</td>
<td>Fig.9</td>
<td>88.60</td>
<td>11.4</td>
</tr>
<tr>
<td>3</td>
<td>3% H2O2 80ºC 1h</td>
<td>4 (0.03, 0.10, 0.50, 0.93)</td>
<td>Fig.10</td>
<td>87.8</td>
<td>12.2</td>
</tr>
<tr>
<td>4</td>
<td>Thermal 100ºC 1h</td>
<td>4 (0.03, 0.08, 0.17, 0.86)</td>
<td>Fig.11</td>
<td>93</td>
<td>7.0</td>
</tr>
<tr>
<td>5</td>
<td>UV 254nm 24h</td>
<td>5 (0.03, 0.26, 0.37, 0.62, 0.68)</td>
<td>Fig.12</td>
<td>88.22</td>
<td>11.78</td>
</tr>
</tbody>
</table>

Fig. 12. Chromatogram for photochemical induced degradation of bergapten.

The degradation study revealed possible number of degradation products from acid, alkali, oxidative, thermal and photolytic degradation. It was observed that overall recovery of drug was good. It was observed that drug exhibited good recovery after acid and thermal degradation than that of other degradation methods.

5. CONCLUSION

The developed stability-indicating HPTLC method for the analysis of bergapten was found simple, precise, accurate and specific. The method was quite sensitive. This method would be helpful in the analysis of bergapten and its formulations. Degradation study has given directions about formulation, packaging and storage conditions. This method can be extended to structure elucidation of degradants products of bergapten by LC-MS.

ACKNOWLEDGMENT

The authors wish to thank Dr. D. Y. Patil Institute of Pharmaceutical Sciences and Research, Pimpri, Pune, India, for providing the facility of camag HPTLC instrumentation and to Dr. K. R. Khandelwal and Dr. Atul Sayare, J.S.P.M’s Rajarshi Shahu College of Pharmacy and Research, Pune, India.

DECLARATION OF INTEREST

The authors declare that there is no conflict of interest.

REFERENCES