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

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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, Apiaceae, 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_f 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 ºC 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 ºC 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 ºC 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 ºC 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 ºC-190 ºC

4.1.2. IR spectrometry

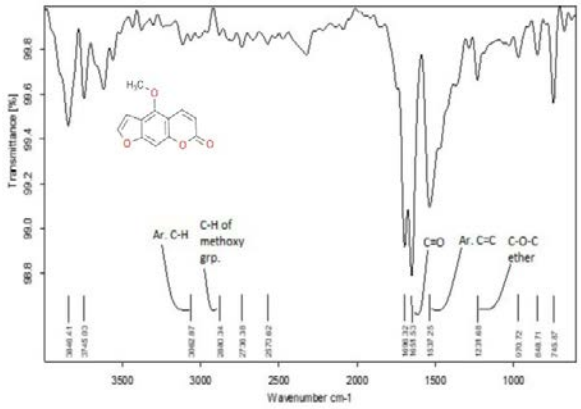

Fig. 1. Infrared spectrum of bergapten.

Table 1. Spectral characterization.

Wavenumber cm^{-1}	Functional groups
3071	Ar. C-H
2880	C-H of the methoxy group
1651	C=O group
1537	Ar. C=C
1232	C-O-C ether

4.1.3. Absorption maxima of bergapten

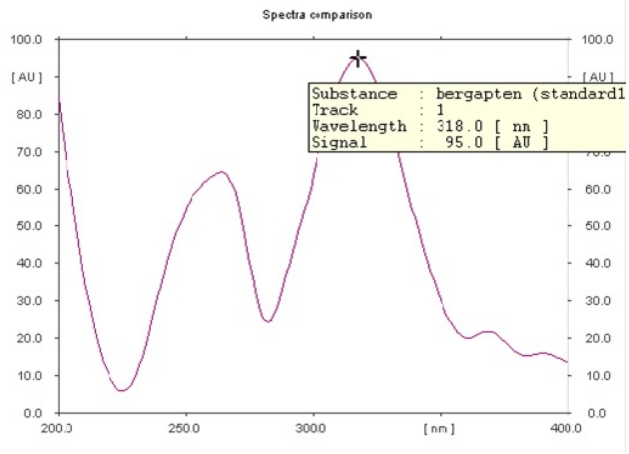

Fig. 2. Absorption maxima of bergapten.

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.

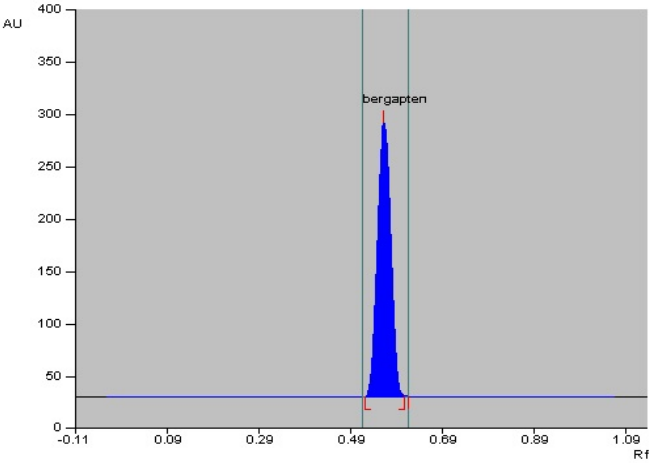

Fig. 3. Chromatogram of bergapten, mobile phase-toluene: dichloromethane: ethyl acetate (7:2:1 v/v/v) and R_f 0.56.

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

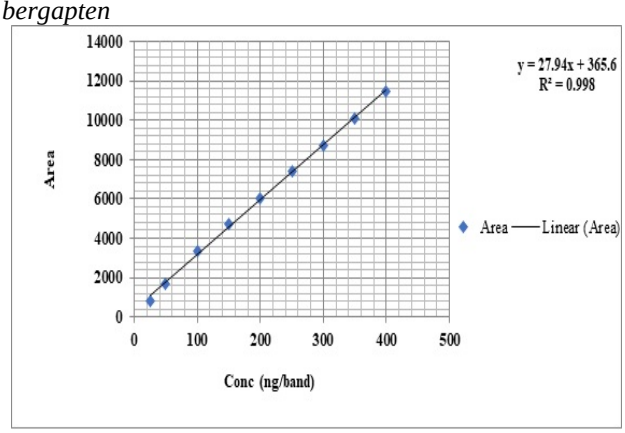

Fig. 4. Calibration curve of bergapten, analytical range-25-400 ng/band, at 318 nm wavelength.

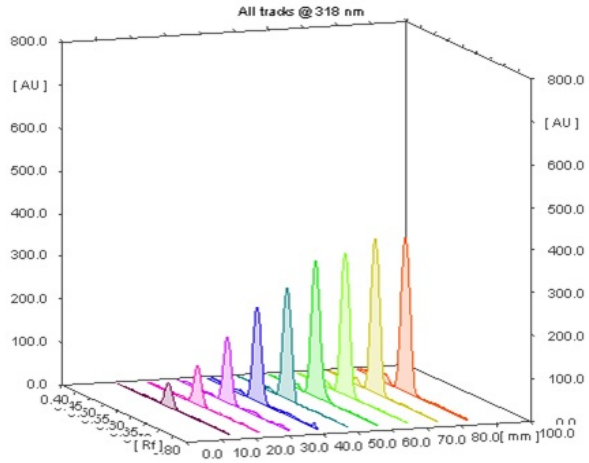

Fig. 5. Three dimensional graph of bergapten for linearity study.

Table 2. Range and linearity data

Parameters	Results
Linearity range (ng/band)	25-400
Correlation coefficient (r^2)	0.998
Slope	27.94
Y Intercept	365.6

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).

Conc. (ng/band)	Mean* conc.(ng)	% found ±SD	RSD (%)
150	149.3	99.57% ±0.277555	0.278732

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

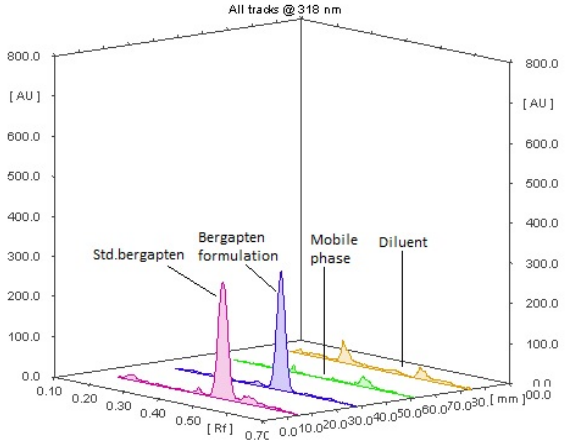

Fig. 6. Three dimensional graph for specificity study.

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

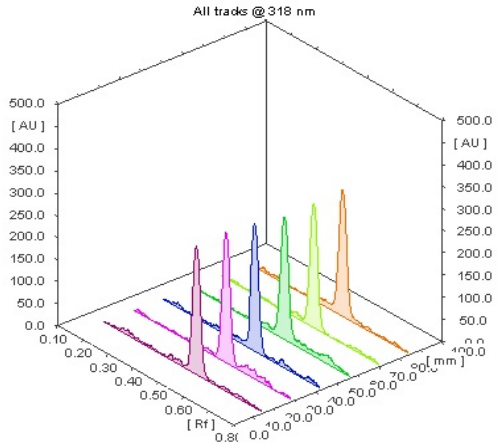

Fig. 7. Three dimensional graph for system precision.

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

Conc.(ng/band)	Mean area	SD	RSD (%)
150	4555.24	10.00	0.21

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. Method precision.

Intra-day precision (*n=3)					Inter-day precision (*n=3)				
Set	Conc. (ng/band)	Mean conc.(ng)	SD	RSD (%)	Set	Conc. (ng/band)	Mean conc.(ng)	SD	RSD (%)
	150	149.8	0.60	0.40		150	149.3	1.67	0.78
0h	200	199.9	1.82	0.91	Day 1	200	201.2	0.45	0.22
	250	249.9	0.25	0.10		250	250.3	0.30	0.12
	150	149.5	0.40	0.27		150	149.8	0.75	0.50
3h	200	198.9	0.84	0.42	Day 2	200	200.7	0.45	0.22
	250	249.5	1.21	0.48		250	249.5	0.40	0.16
	150	149.6	0.05	0.03		150	150.0	0.40	0.26
6 h	200	200.7	1.05	0.52	Day 3	200	198.8	0.89	0.45
	250	249.2	0.98	0.39		*250*	250.1	0.30	0.11

Table 6. Recovery studies (*n=3).

Levels of Standard addition (%)	Theoretical content (ng)	Mean Recovery (%)	SD	RSD (%)
80	270	99.78	0.75	0.28
100	300	99.85	0.92	0.31
120	330	99.79	0.64	0.19

4.3.5. Robustness of the method

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

Conc.(ng/band)	Parameters		R_f
150	Optimized mobile phase	Toluene: dichloromethane: ethyl acetate (7:2:1 v/v/v)	0.56
150	Mobile phase composition	Toluene: dichloromethane: ethyl acetate (8:1:1 v/v/v)	0.53
150		Toluene: dichloromethane: ethyl acetate (6:3:1 v/v/v)	0.54
150	Mobile phase saturation time	5 minutes	0.57
150		15 minutes	0.53

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.

Parameters	Concentration
LOD	10.17 ng
LOQ	30.82 ng

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

4.4. Forced degradation study

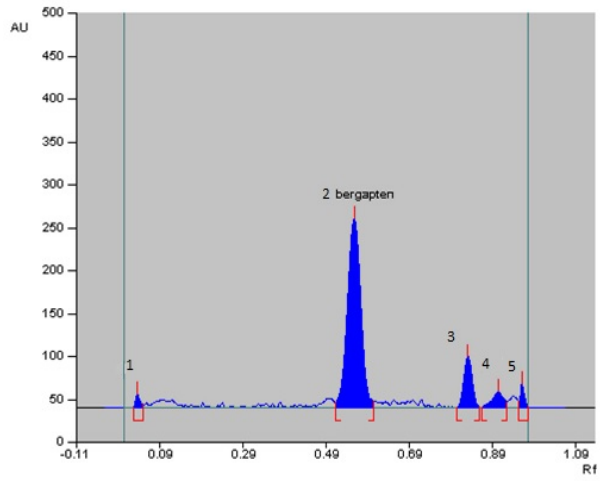

Fig. 8. Chromatogram for acid induced degradation of bergapten.

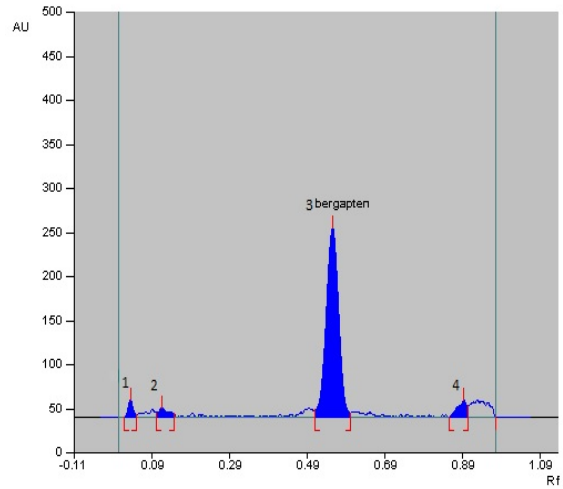

Fig. 9. Chromatogram for alkali induced degradation of bergapten.

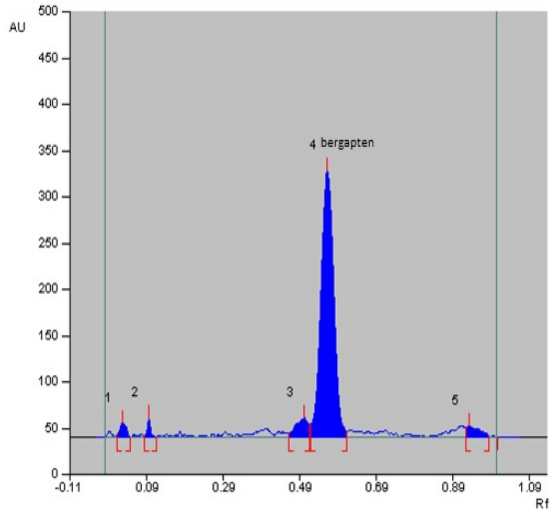

Fig. 10. Chromatogram for hydrogen peroxide induced degradation of bergapten.

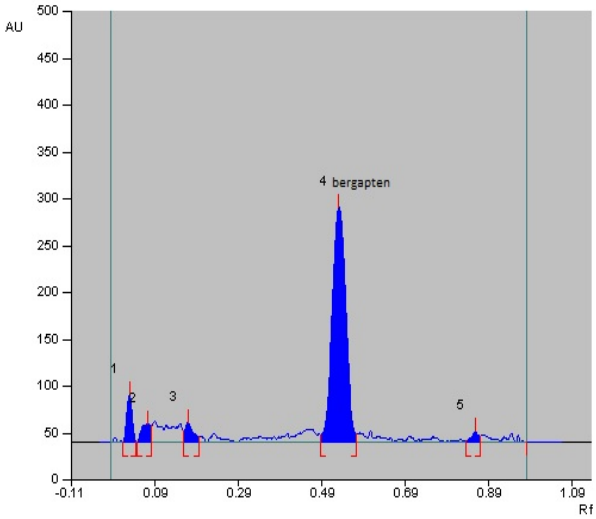

Fig. 11. Chromatogram for dry heat induced degradation of bergapten.

Table 9. Forced degradation of bergapten.

Sr. No.	Forced degradation condition	Number of degradation products (R_f value)	Figure	% Drug recovered	% Drug degraded
1	1N HCL 80ºC 1h	4 (0.04, 0.83, 0.90, 0.96)	Fig.8	91.16	8.8
2	0.1N NaOH 80ºC 2h	3 (0.03, 0.12, 0.89)	Fig.9	88.60	11.4
3	3% H_2O_2 80ºC 1h	4 (0.03, 0.10, 0.50, 0.93)	Fig.10	87.8	12.2
4	Thermal 100ºC 1h	4 (0.03, 0.08, 0.17, 0.86)	Fig.11	93	7.0
5	UV 254nm 24h	5 (0.03, 0.26, 0.37, 0.62, 0.68)	Fig.12	88.22	11.78

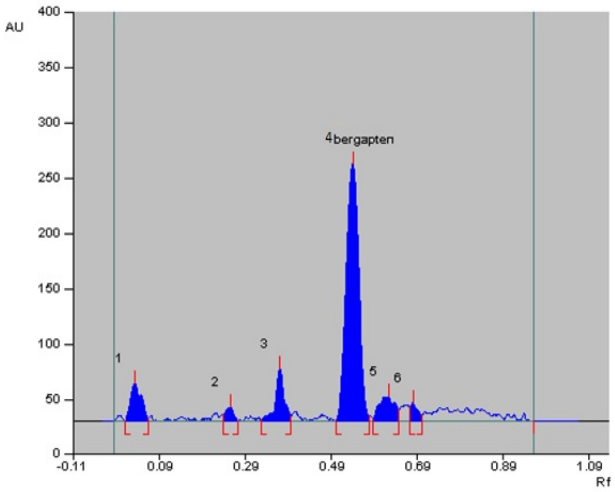

Fig. 12. Chromatogram for photochemical induced degradation of bergapten.

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.

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Declaration Of Interest

The authors declare that there is no conflict of interest.

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