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HPTLC method for guggulsterone II. Stress degradation studies on guggulsterone

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Abstract

Stress degradation studies were carried out on guggulsterone (the hypolipidemic agent in the gum-resin exudates of *Commiphora mukul*) following the conditions prescribed in the parent drug stability testing guideline (Q1AR) issued by International Conference on Harmonization (ICH). The present study describes degradation of guggulsterone under different ICH prescribed stress conditions (acid and base hydrolysis, oxidation, dry and wet heat degradation and photodegradation) and establishment of a stability indicating HPTLC assay. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene–acetone (9:1, v/v). Densitometric analysis of guggulsterone was carried out in the absorbance mode at 250 nm. This system was found to give compact spots for E- and Z-guggulsterone, (R_f value of 0.38 ± 0.02 and 0.46 ± 0.02, respectively) following double development of chromatoplates with the same mobile phase. The drug undergoes degradation under acidic and basic conditions, oxidation, dry and wet heat treatment and photodegradation. All the peaks of degraded products were resolved from the standard guggulsterone with significantly different R_f values. As the method could effectively separate the drug from its degradation products, it can be employed as a stability indicating one.

Keywords: Guggulsterone; *Commiphora mukul*; HPTLC; Stability indicating; Stress degradation

1. Introduction

The parent drug stability test guidelines (Q1A) issued by International Conference on Harmonization (ICH) requires that analytical test procedures for stability samples should be fully validated and the assays should be stability indicating [1]. Stress testing is a part of development strategy under ICH requirements and is carried out under more severe conditions than accelerated studies. Further, it is suggested that stress studies should be carried out to establish the inherent or intrinsic stability characteristics of the molecule by establishing the degradation pathways and help in validation of the analytical methods to be used in stability studies. The parent drug stability guidelines (Q1AR) requires that stress testing of drug substance should include the effect of elevated temperature, humidity, light and oxidizing agents, as well as the susceptibility across a range of pH values [2].

Accordingly, the purpose of the present study is to put ICH recommendations into practice by subjecting guggulsterone to the variety of suggested stress test conditions to establish inherent stability of the drug and to develop the validated stability indicating HPTLC assay. The endeavor was to quantify overall amount of degradation of E- and Z-guggulsterone under different stress conditions. There is no report yet on these aspects for this drug. Guggulsterone (Fig. 1) is chemically {4,17(20)-pregnadiene-3,16-dione}. The cholesterol reducing activity can be traced to the two closely related steroidal ketones, Z-{4,17(20)-*cis*-pregnadiene-3,16-dione,1a} guggulsterone and E-{4,17(20)-*trans*-pregnadiene-3,16- dione,1b} guggulsterone. Literature survey reveals spectrophotometric assay ($\lambda = 327$) [3] and HPLC methods for the determination of guggulsterone in biological fluids [4–7]. Simultaneous determination of stereo isomers of guggulsterone in serum by HPLC [8] and HPLC method for fingerprinting

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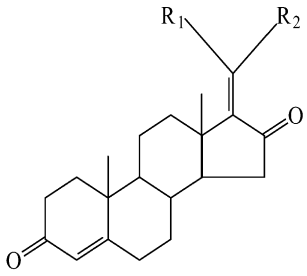

	R_1, R_2	Compound	Absolute Configuration
1a	$R_1 = H, R_2 = CH_3$	Guggulsterone	Z –
1b	$R_1 = CH_3, R_2 = H$	Guggulsterone	E –

Fig. 1. Chemical structure of guggulsterone (1), Z-isomer (1a) and E-isomer (1b).

and quantitative determination of E- and Z-guggulsterone in *Commiphora mukul* and its products have been reported [9]. In a previous study (part I), we developed and validated HPTLC assay for guggulsterone isomers in herbal extract and pharmaceutical dosage form. In this study, we investigated the effect of different stress conditions on the stability of the drug with the help of previously described validated HPTLC method. The International Conference on Harmonization (ICH) guidelines entitled 'stability testing of new drug substances and products' require the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance. Susceptibility to oxidation is one of the required tests. Also the hydrolytic and the photolytic stability are required. An ideal stability indicating method is one that quantifies the standard drug alone and also resolves its degradation products. Ferenczi-Fodor et al. [10,11] explained basic acceptance criteria for evaluation of validation experiments based on practical experience for planar chromatographic procedures, which may be used at different levels either in qualitative identity testing, assays, semi-quantitative limit tests or quantitative determination of impurities. The parameters for robustness testing of given procedures and quality assurance of quantitative planar chromatographic testing have been described as per International Conference on Harmonization (ICH) guidelines. According to the European pharmacopoeia [12], the profile of the impurities has been defined in relation to the sources of drug identified. The impurities detected by HPTLC are limited to 0.1 per cent. The limits for these impurities have been fixed at the minimum level permitted by the analytical method in accordance with the requirements laid down in system conformity.

The aim of the present work is to employ validated HPTLC method for stability studies of E- and Z-isomers of guggulsterone in presence of its degradation products and related impurities for assessment of purity of bulk drug and stability of its dosage forms. Analysis of guggulsterone in marketed products has shown surprising results. Mesorb et al. [9] have reported very less amount of guggulsterone both in extract and marketed formulations. The content of E- and Z-guggulsterone in these materials varied widely and was found significantly less than claimed for both of the guggulsterones. In the last few years the number of guggulsterone products in the market has decreased significantly. Therefore it is necessary to design a stability indicating HPTLC method.

Table 1
Stability of (a) E-isomer and (b) E-isomer of guggulsterone in sample solutions ($n = 6$)

Actual (ng/spot)	Area mean	Area range	R.S.D. (%)	S.E.
(a) Stability of E-isomer				
400	4890.56	4872.23–4901.12	1.78	1.23
800	9881.12	9868.35–9898.29	1.85	1.42
(b) Stability of Z-isomer				
400	4972.48	4965.56–4988.56	1.56	1.01
800	9984.96	9973.58–9992.69	1.75	1.22

2. Experimental

2.1. Materials

Standard guggulsterone mixture (containing E- and Z-isomers, purity 99.17%, w/w), standard E-isomer (purity 99.34%, w/w) and standard Z-isomer of guggulsterone (pu-

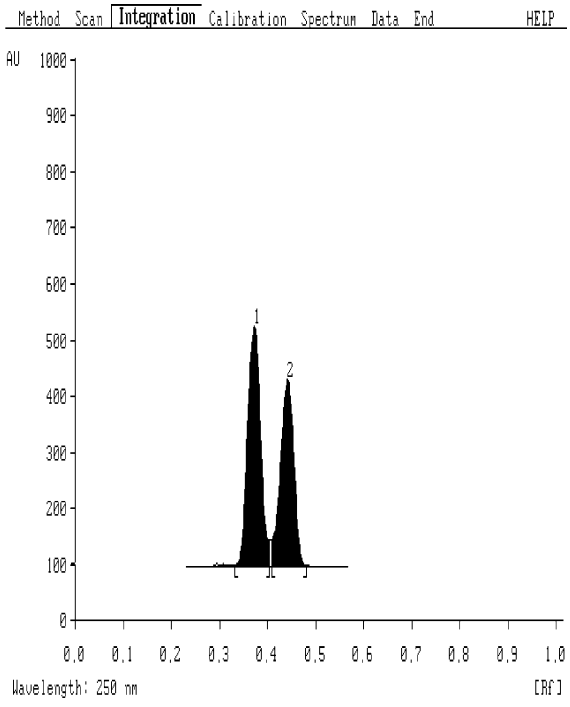

Fig. 2. Chromatogram of standard guggulsterone mixture (1000 ng/spot). Peak 1 is of E-guggulsterone (R_f: 0.38 ± 0.02), peak 2 is of Z-guggulsterone (R_f: 0.46 ± 0.02); mobile phase; toluene–acetone (9.0: 1.0, v/v).

rity 99.07%, w/w) were kindly provided as a gift sample by Laila Impex, Vijaywada, India and were used without further purification. Guggulu extract and guggulu capsules were procured from the local market. All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

2.2. HPTLC instrumentation

The samples were spotted in the form of bands of width 6 mm with a Camag microlitre syringe on precoated silica gel aluminium plate 60 F–254, (20 cm × 10 cm with 250 μm thickness; E. Merck, Darmstadt, Germany, supplied by Anchrom Technologists, Mumbai) using a Camag Linomat IV (Switzerland). The plates were prewashed by methanol and activated at 60 °C for 5 min prior to chromatography. A constant application rate of 0.1 μl/s was employed and space between two bands was 5 mm. The slit dimension was kept at 5 mm × 0.45 mm and 10 mm/s scanning speed was employed. The monochromatic bandwidth was set at 20 nm, each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene–acetone (9: 1, v/v) and 15 ml of mobile phase was used per chromatography. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (Camag, Muttenz, Switzerland) saturated with the mobile phase and the chromatoplate development was carried out for two times with the same mobile phase to get good resolution of E- and Z-isomers of guggulsterone. The optimized chamber saturation time for mobile phase was 30 min at room temperature (25 ± 2 °C) at relative humidity of 60 ± 5%. The length of chromatogram run was 8 cm. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on Camag TLC scanner III in the reflectance-absorbance mode at 250 nm and operated by CATS software (V 3.15, Camag). The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 190 and 400 nm. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak areas with linear regression.

2.3. Forced degradation of standard guggulsterone

A stock solution containing 100 mg of standard guggulsterone containing E- and Z-isomers in 100 ml methanol was prepared. This solution (1 mg/ml) was used for forced degradation.

2.3.1. Preparation of acid and base-induced degradation products

For acid degradation studies, to 5 ml of methanolic stock solution, 5 ml each of 1- and 5N HCl were added separately. These mixtures were refluxed for 1.0 and 2.0 h at 80 °C. For degradation studies in alkaline conditions, to 5 ml of methanolic stock solution, 5 ml each of 1- and 5N NaOH

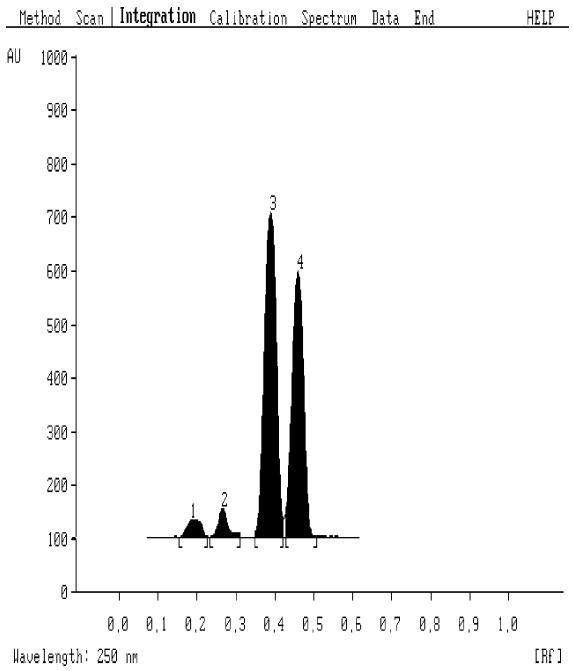

Fig. 3. Chromatogram of acid (5N HCl, reflux for 1.0 h, temperature 80 °C) treated standard guggulsterone mixture (2000 ng/spot). Peak 1 (degraded) (R_f: 0.20), peak 2 (degraded) (R_f: 0.27), peak 3 (E-guggulsterone) (R_f: 0.38 ± 0.02), peak 4 (Z-guggulsterone) (R_f: 0.46 ± 0.02).

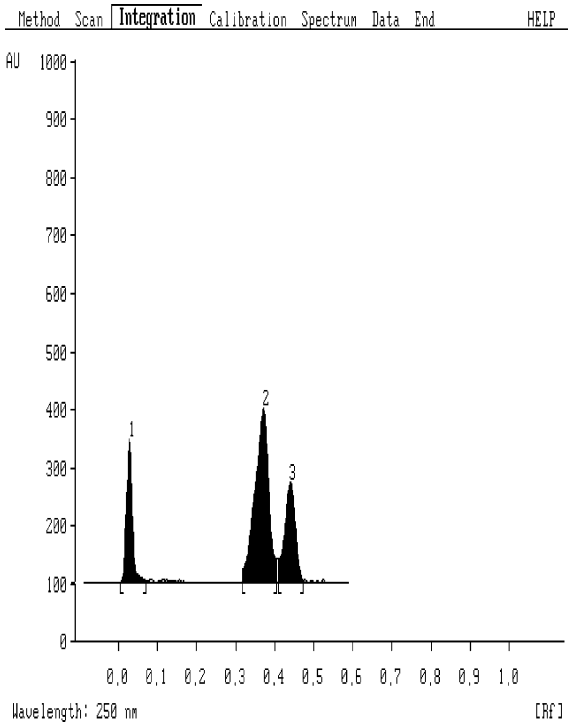

Fig. 4. Chromatogram of base (1N NaOH, reflux for 1.0 h, temperature 80 °C) treated standard guggulsterone mixture (1000 ng/spot). Peak 1 (degraded) (R_f: 0.03), peak 2 (E-guggulsterone) (R_f: 0.38 ± 0.02), peak 3 (Z-guggulsterone) (R_f: 0.46 ± 0.02).

were added separately. These mixtures were refluxed for 1.0 and 2.0 h at 80 °C. To study the degradation of drug in phosphate buffer pH 8.0, 5 ml of buffer solution was added to 5 ml of methanolic stock solution. It was refluxed at 80 °C for 2 h. The forced degradation in acidic and basic media was performed in the dark in order to exclude the possible degradative effect of light. Two microlitres of the resultant solutions (1000 ng/spot) were applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.3.2. Preparation of hydrogen peroxide-induced degradation product

For oxidative conditions, initially to 5 ml of methanolic stock solution, 15 ml of 6.0% (w/v) hydrogen peroxide was added. Subsequently, to the 15 ml of methanolic stock solution 5 ml of 50.0% (w/v) hydrogen peroxide was added. The solutions were heated in boiling water bath for 10 min to remove completely the excess of hydrogen peroxide and then refluxed for 2.0 h at 80 °C. Four microlitres (1000 ng/spot) and two microlitres (1500 ng/spot) of the resultant solutions were applied on TLC plate and the chromatograms were run as described in Section 2.2.

2.3.3. Dry and wet heat degradation product

The standard guggulsterone powder was stored in oven at 90 °C for 4 h to study dry heat degradation and the stock solution was refluxed for 2.0 h on boiling water bath for wet heat degradation.

2.3.4. Photochemical degradation product

The photochemical stability of the drug was also studied by exposing the stock solution to direct sunlight for three days (from 09:00 to 17:00 h at ≈30 °C) on a wooden plank and kept on terrace. One microlitre of the solution (1000 ng/spot) was applied on TLC plate and chromatograms were run as described in Section 2.2.

In all degradation studies the average peak area of E- and Z-isomers of guggulsterone after application of seven replicates were obtained.

2.4. Detection of the related impurities of E- and Z-isomers of guggulsterone

The related impurities were determined by spotting higher concentrations of the E- and Z-isomers so as to detect and quantify them. E- and Z-isomers 10 mg each, were dissolved in 5 ml of methanol and these solutions were termed as E- and Z-sample solutions (2000 ng/μl). One ml of each sample solution was diluted to 10 ml with methanol and these solutions were termed as E- and Z-standard solutions (200 ng/μl). One microlitre of each standard solution (200 ng/spot) and ten microlitres of each sample solutions

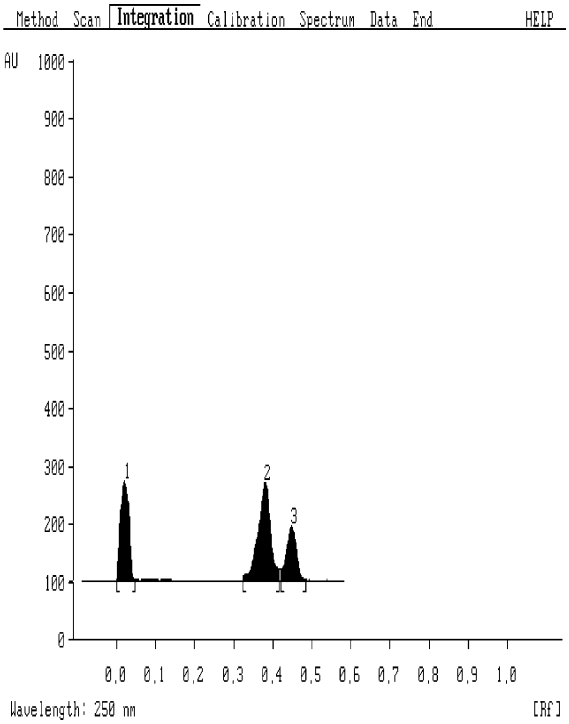

Fig. 5. Chromatogram of phosphate buffer pH 8 (refluxed for 2.0 h, temperature 80 °C) treated standard guggulsterone mixture (1000 ng/spot). Peak 1 (degraded) (R_f: 0.02), peak 2 (E-guggulsterone) (R_f: 0.38 ± 0.02), peak 3 (Z-guggulsterone) (R_f: 0.46 ± 0.02).

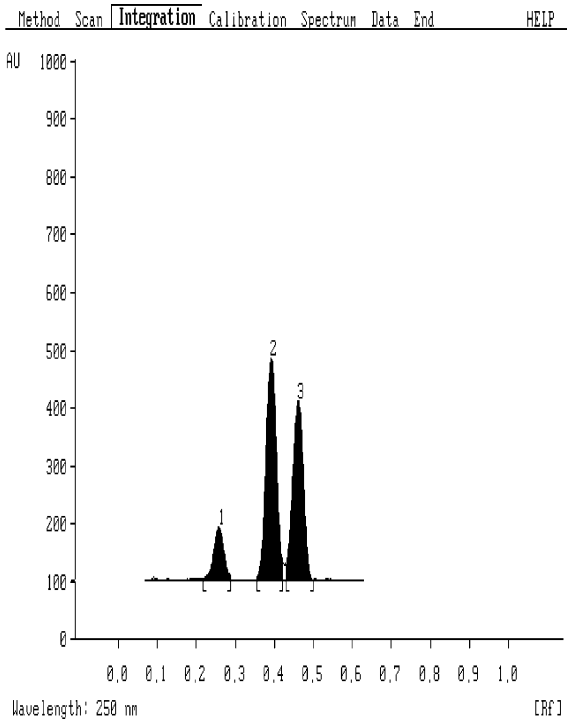

Fig. 6. Chromatogram of hydrogen peroxide (6% w/v, reflux for 2.0 h, temperature 80 °C) treated standard guggulsterone mixture (1000 ng/spot). Peak 1 (degraded) (R_f: 0.26), peak 2 (E-guggulsterone) (R_f: 0.38 ± 0.02), peak 3 (Z-guggulsterone) (R_f: 0.46 ± 0.02).

(20,000 ng/spot) were applied on TLC plate and the chromatograms were run as described in Section 2.2.

3. Results and discussion

3.1. Stability of E- and Z-isomers in sample solution

Solutions of two different concentrations (400 and 800 ng/spot) were prepared from sample solution and stored at room temperature for 6.0, 12.0, 24.0, 48.0 and 72.0 h, respectively. The solutions were stored in tightly capped volumetric flasks protected from light on a laboratory bench. They were then applied on the same TLC plate, after development the chromatogram was evaluated as listed in Table 1 for additional spots if any. The R.S.D. (%) for the samples analysed at different elapsed assay times was found to be <2%. Thus the drug was stable in solution state. There was no indication of compound instability in the sample solution.

3.1.1. Spot stability of E- and Z-isomers

The time the sample is left to stand on the solvent prior to chromatographic development can influence the stability of separated spots and are required to be investigated for validation [13]. Two-dimensional chromatography using same solvent system was used to find out any decomposition occurring during spotting and development. In case, if decomposition occurs during development, peak(s) of decomposition product(s) shall be obtained for the analyte both in the first and second direction of the run. No decomposition was observed during spotting and development (Fig. 2).

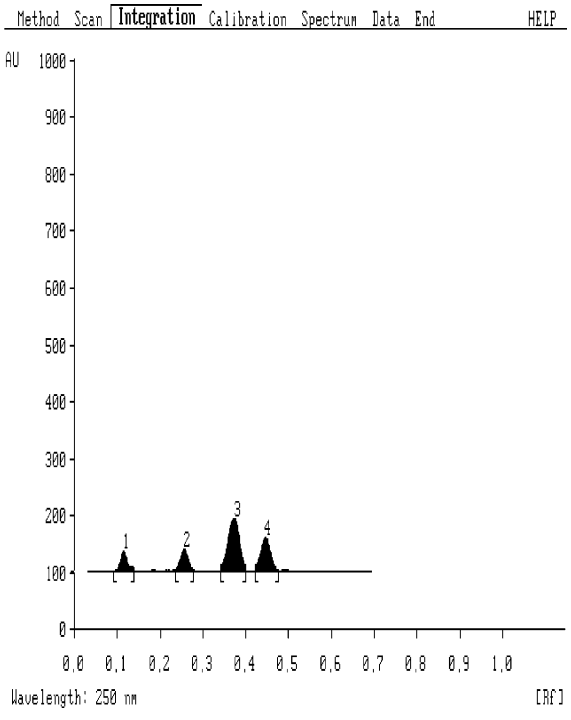

Fig. 7. Chromatogram of hydrogen peroxide (50% w/v, reflux for 2.0 h, temperature 80 °C) treated standard guggulsterone mixture (1000 ng/spot). Peak 1 (degraded) (R_f: 0.12), peak 2 (degraded) (R_f: 0.26), peak 3 (E-guggulsterone) (R_f: 0.38 ± 0.02), peak 4 (Z-guggulsterone) (R_f: 0.46 ± 0.02).

3.2. Stability indicating property

3.2.1. Acid and base-induced degradation product

The drug gets slowly degraded in strongly acidic conditions. On refluxing at 80 °C in 1 N HCl (1, 2 h), 5N HCl (1, 2 h), the area of the drug peaks decreased and two peaks were generated at R_f 0.20 and 0.27 (Fig. 3). In alkali, the drug was found to decompose rapidly. On refluxing at 80 °C in 1N NaOH (1, 2 h), 5N NaOH (1, 2 h), showed additional peak at R_f value of 0.03 (Fig. 4). The reaction in 5N NaOH at 80 °C was so fast that whole of the drug was degraded in two hours. Subsequent studies were performed in milder conditions in phosphate buffer pH 8 at 80 °C. The area of the drug peaks decreased with corresponding rise in new peak at R_f 0.02 (Fig. 5). The areas of the degraded peaks were found to be lesser than the area of standard drug concentration (1000 ng/spot) indicating that guggulsterone undergoes degradation under acidic and basic conditions.

3.2.2. Hydrogen peroxide-induced degradation product

The sample degraded with 6.0% (w/v) hydrogen peroxide showed additional peak at R_f value of 0.26 (Fig. 6). The sample degraded with 50.0% (w/v) hydrogen peroxide

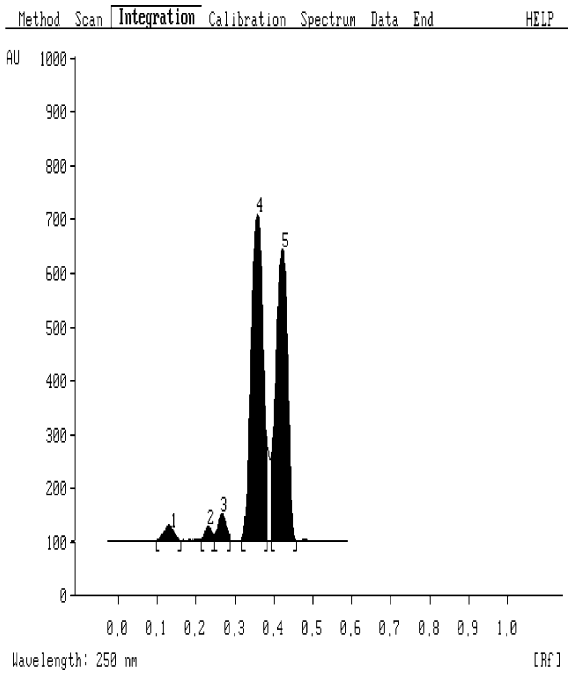

Fig. 8. Chromatogram of dry heat (90 °C for 4 h) treated standard guggulsterone mixture (1000 ng/spot). Peak 1 (degraded) (R_f: 0.14), peak 2 (degraded) (R_f: 0.23), peak 3 (degraded) (R_f: 0.27), peak 4 (E-guggulsterone) (R_f: 0.37 ± 0.02), peak 5 (Z-guggulsterone) (R_f: 0.44 ± 0.02).

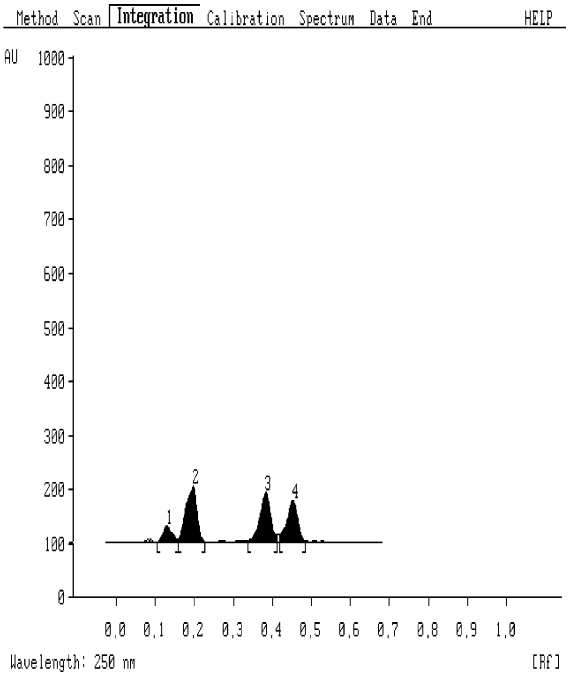

Fig. 9. Chromatogram of wet heat (refluxed at 100 °C for 2 h) treated standard guggulsterone mixture (1000 ng/spot). Peak 1 (degraded) (R_f: 0.13), peak 2 (degraded) (R_f: 0.19), peak 3 (E-guggulsterone) (R_f: 0.38 ± 0.02), peak 4 (Z-guggulsterone) (R_f: 0.45 ± 0.02).

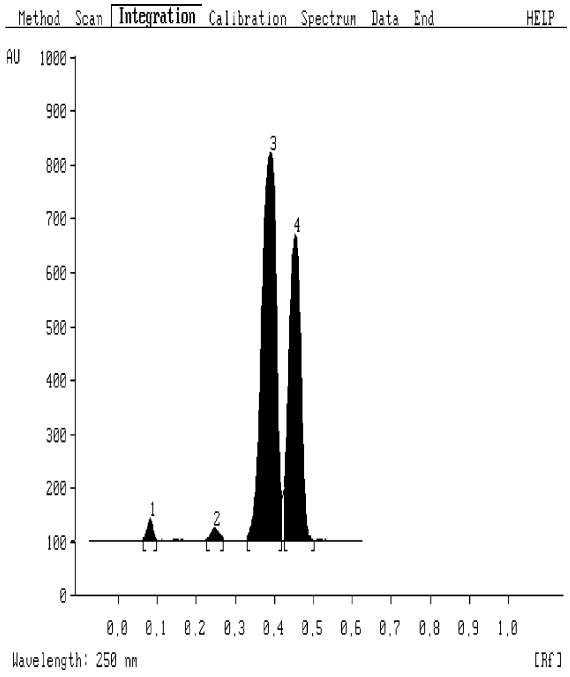

Fig. 10. Chromatogram of photo degraded standard guggulsterone mixture kept in sun light for 24 h (1000 ng/spot). Peak 1 (degraded) (R_f: 0.08), peak 2 (degraded) (R_f: 0.25), peak 3 (E-guggulsterone) (R_f: 0.38 ± 0.02), peak 4 (Z-guggulsterone) (R_f: 0.46 ± 0.02).

showed two additional peaks at R_f value of 0.26 and 0.12 (Fig. 7). The spots of degraded products were well resolved from the drug spot.

3.2.3. Dry and wet heat degradation product

The samples degraded under dry heat condition showed three additional peaks at R_f values of 0.14, 0.23, 0.27 (Fig. 8) and wet heat conditions showed two additional peaks at R_f values of 0.13, 0.19 (Fig. 9).

3.2.4. Photochemical degradation product

The photo degraded sample showed two additional peaks at R_f 0.08 and 0.25 when methanolic solution of standard guggulsterone (1000 ng/μl) was exposed to sunlight for 24 h (Fig. 10).

This indicates that the E- and Z-isomers of guggulsterone are susceptible to acid and base hydrolysis, oxidation, dry and wet heat degradation and photodegradation. The lower R_f values of acid degraded, base degraded, dry and wet heat degraded and photo degraded components indicated that they were more polar than the analyte itself. The results are listed in Table 2.

3.3. Detection of the related impurities

The spots other than the principal spot and the spot of the starting point from the sample solution were not intense than

Table 2
Stress degradation studies of E- and Z-guggulsterone

Condition	Time (h)	% Recovery		R_f value of degradation products
		E-isomer	Z-isomer	
Acid 1N HCl, ref[a]	1.0	97.65	96.13	0.20, 0.27
Acid 5N HCl, ref	1.0	95.96	94.23	0.20, 0.27
Base 1N NaOH, ref	1.0	96.34	51.41	0.03
Base 1N NaOH, ref	2.0	91.12	36.62	0.03
Base 5N NaOH, ref	1.0	0.0	0.0	0.03
Phosphate buffer pH 8, ref	2.0	50.04	26.47	0.02
H_2O_2 6.0% (w/v), ref	2.0	93.04	94.13	0.26
H_2O_2 50.0% (w/v), ref	2.0	16.05	11.15	0.12, 0.26
Dry heat (90 °C)	4.0	44.49	46.54	0.14, 0.23, 0.27
Wet heat (100 °C)	2.0	21.60	22.50	0.13, 0.19
Day light (25 °C)	24.0	48.08	38.39	0.08, 0.25

[a] Refluxed at 80 °C.

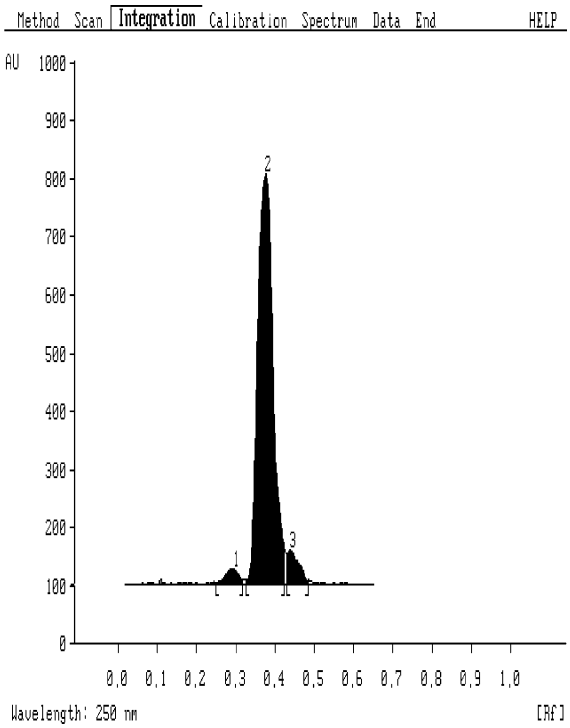

Fig. 11. Chromatogram of standard E-isomer of guggulsterone and its impurities. Peak 1 (impurity) (R_f: 0.28), peak 2 (standard E-guggulsterone) (R_f: 0.38), peak3 (impurity) (R_f: 0.43).

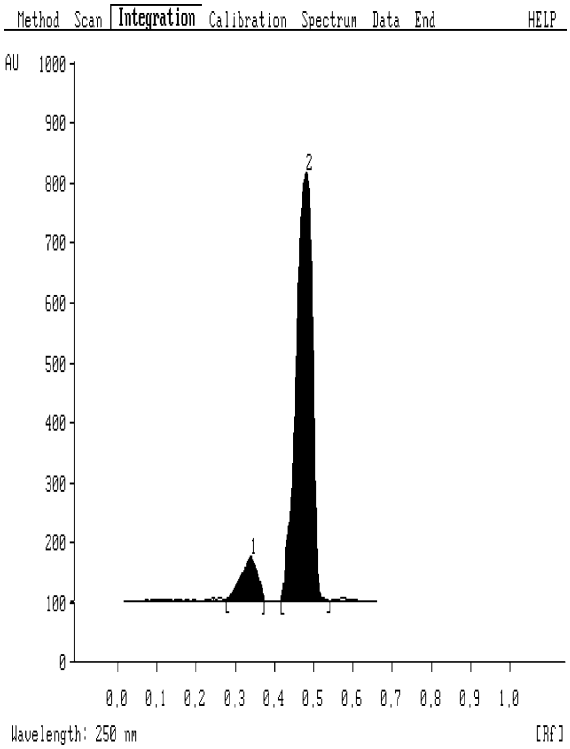

Fig. 12. Chromatogram of standard Z-isomer of guggulsterone and its impurity. Peak 1 (impurity) (R_f: 0.33), peak 2 (standard Z-guggulsterone) (R_f: 0.47).

Table 3
Related impurities of (a) E-isomer and (b) Z-isomer of guggulsterone

Concentration of drug (ng/spot)	R_f value	Area
E-isomer		
200	0.38	2445.28
Related impurity		
20,000	0.28	827.8
	0.43	1203.9
Total area		2031.7
Z-isomer		
200	0.46	2486.24
Related impurity		
20,000	0.33	1755.33

the spot from the standard solution. The standard E-isomer of guggulsterone solution showed two additional spots at R_f 0.28 and 0.43 (Fig. 11). The standard Z-isomer of guggulsterone solution showed one additional spot at R_f 0.33 (Fig. 12). However the area of the additional spots were found to be much less as compared to the standard solution as indicated in Table 3.

Degradation of drug due to oxidation (50%, w/v H_2O_2), dry and wet heat degradation has led to formation of a common degradation entity with R_f in the range of 0.12–0.14. Presence of the same component was also observed in the chromatogram of extract (peak 1) and marketed capsule (peak 2). Degradation of drug due to acid hydrolysis,

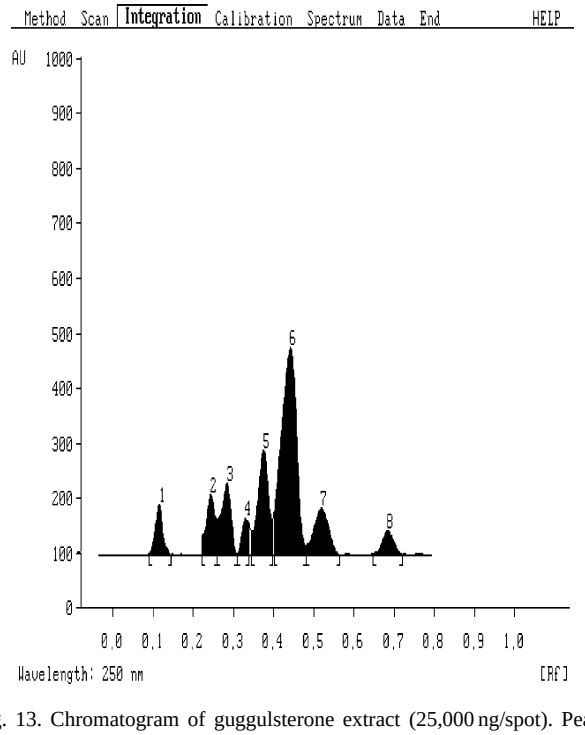

Fig. 13. Chromatogram of guggulsterone extract (25,000 ng/spot). Peaks 1, 2, 3, 4, 7 and 8 belongs to other components present in the extract. Peak 5 is of E-guggulsterone (R_f: 0.38 ± 0.02), peak 6 is of Z-guggulsterone (R_f: 0.46 ± 0.02); mobile phase; toluene–acetone (9.0:1.0, v/v).

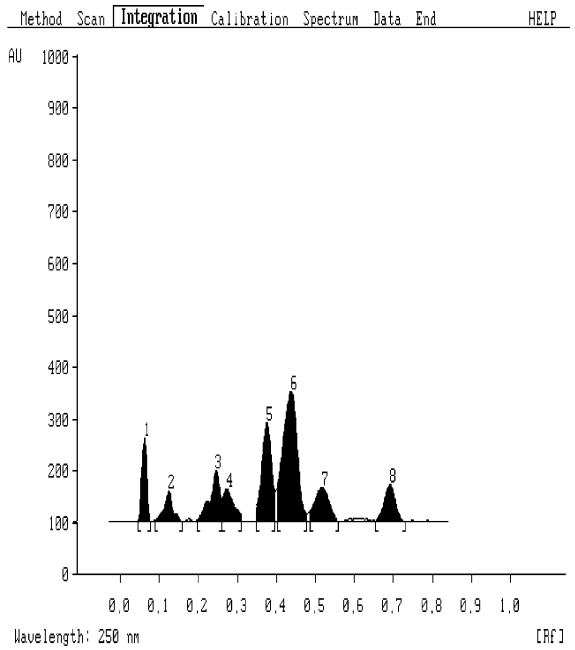

Fig. 14. Chromatogram of guggulu capsule (25,000 ng/spot). Peaks 1, 2, 3, 4, 7 and 8 belongs to other components present in the capsule. Peak 5 is of E-guggulsterone (R_f: 0.38 ± 0.02), peak 6 is of Z-guggulsterone (R_f: 0.46 ± 0.02); mobile phase; toluene–acetone (9.0:1.0, v/v).

oxidation, dry heat and photo degradation yielded product with R_f value in the range of 0.25–0.27 which can be correlated with chromatogram of extract (peaks 2 and 3) and capsule (peaks 3 and 4) Apart from this the photo degraded product with R_f 0.08 was observed in chromatogram of marketed preparation but absent in extract. The impurity of E-isomer at R_f 0.28 can be attributed due to acid hydrolysis or dry heat degradation. The impurity of Z-isomer at R_f 0.33 can be correlated with peak 4 (R_f = 0.32) in the chromatogram of extract (Fig. 13) but absent in capsule (Fig. 14). Thus only the peak at R_f 0.54 and 0.70 observed in the marketed capsule and extract did not match with any of the peaks in forced degraded products. Thus it can be considered that most of the impurities present in the marketed preparations may have been formed due to degradation during processing and storage.

Preparation of dried extract from the resin involves various extraction and drying steps. It involves high temperature processing at preformulation stage and subjected to significantly high temperatures (up to 140 °C) [14] at the final drying stage. Thus there are high chances of degradation during these stages. But some peaks that have not been attributed to any of the degradation products may be due to contamination during collection or other source or impurity produced by some other route. Development of impurity profile and strict adherence to quality assurance steps may aid in improving product quality.

4. Conclusion

This study is a typical example of development of a stability-indicating assay, established following the recommendations of ICH guidelines. It is one of the rare studies where forced decomposition studies were done under all different suggested conditions and the degradation products were well resolved. The HPTLC technique employed is stability indicating since it resolves all the degradation products of guggulsterone under all stress conditions. This study may be extended to study the degradation kinetics of guggulsterone and to predict degradation pathways. This method can be proposed for the analysis of guggulsterone and its degradation products in stability samples in industry. A new finding of this study is that the guggulsterone is unstable in almost all forced degradation conditions. As the method separates the drug from its degradation products, it can be employed as a stability indicating one.

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