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Simple Stability-Indicating HPLC Technique for Levamisole and Triclabendazole Combinational Analysis, Evaluation of Stabilities of Levamisole and Triclabendazole in Applied Stress Conditions

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Abstract : For the simultaneous quantitative prediction of LVME and TCBE combination in oral suspension, a "stability indicating HPLC technique" was presented in current work. The accuracy and precision of this "stability indicating HPLC technique" were justified by analyzing LVME and TCBE combo in suspension by using typical addition technique. The findings produced using the "stability indicating HPLC technique" were claimed to have remarkable accuracy and precision. As an outcome, the "stability indicating HPLC technique" may be implemented for regular quality monitoring of LVME and TCBE suspension formulations. For the quantification of LVME and TCBE, a "stability indicating HPLC technique" was created and validated. This method can be successfully applied for LVME and TCBE combination analysis as well as for LVME and TCBE stability study. On the whole, the "stability indicating HPLC technique" provides prominent through put technique for combo determination of LVME and TCBE in Clobend-L suspension and bulk with excellent sensitivity, precision, selectivity, and accuracy.
Key-words : HPLC Technique,Levamisole ,Triclabendazole Combinational Analysis.

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1. Introduction

The combination of levamisole (LVME, see figure 1) and triclabendazole (TCBE, see figure 1) is available in suspension and bolus form on the market[1,2]. This combination is a very much effective nematicide and flukicide. *Fasciola hepatica* as well as *Fasciola gigantica* produce all sorts of fasciolosis, which can be efficiently treated with LVME and TCBE combination[3]. Major ruminant parasites of abomasum (*Ostertagia and Haemonchus*), small intestine (*Trichostrongylus, Cooperia, and Bunostomum*), large intestine (*Trichuris and Oesophagostomum*) and lungs (*Dictyocaulus spp.*) are satisfactorily removed by this LVME and TCBE anthelmintics combination[4,5].

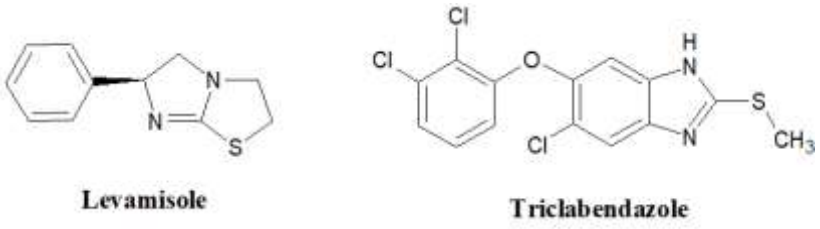

Figure: 1 LVME and TCBE structures

LVME has a direct impact on the neurological system of the worm, paralysing it and separating it from the respiratory as well as gastrointestinal mucosa[6,7]. Since TCBE suppresses the enzyme Fumarate reductase, energy generation in the worm's body is disrupted. The effects of TCBE are felt throughout the parasite's life cycle, from immature to mature phases[8,9].

Gözde et al., developed methods like partial least square, component principal regression, and chemometry to quantify LVME and TCBE combination in oral tablet samples[10]. Other than Gözde et al., techniques, no other methodologies for quantifying the combination of LVME and TCBE has been published. Furthermore, the methodologies used by Gözde et al., were not "stability indicating".

For the simultaneous quantitative prediction of LVME and TCBE combination in oral suspension, a "stability indicating HPLC technique" was presented in current work. The accuracy and precision of this "stability indicating HPLC technique" were justified by analyzing LVME and TCBE combo in suspension by using typical addition technique. The findings produced using the "stability indicating HPLC technique" were claimed to have remarkable accuracy and precision. As an outcome, the "stability indicating HPLC technique" may be implemented for regular quality monitoring of LVME and TCBE suspension formulations.

2. Materials and methods

Instruments

A Waters liquid chromatography equipment was linked to a PDA system (Waters). Waters' HPLC system included an auto sampler, a column oven, a binary pump, and a controller. For device control, data collecting, and processing, Empower software (Waters) version two was employed.

Chemicals

LVME and TCBE were acquired from "Ven life sciences Pvt Ltd, Telangana, India" as gift. Methanol was acquired from "Merck". Sodium hydroxide, peroxide, HCl and Na_2SO_4 were acquired from "Sd fine chemicals Ltd". Clobend-L suspension of LVME (37.5 mg/mL) and TCBE (50 mg/mL) were acquired from Indian drug store marketplace.

Chromatography assaying conditions

Chromatography assaying of LVME and TCBE was accomplished by a 25 cm ×0.46 cm, 5 µm, column Kromasil RP-C18 at a temperature of 25 °C. The solvents of mobile phase included 0.1 M Na_2SO_4, pH 3.5 (60% vol. ratio) and pure methanol (40% vol. ratio). The sample size of injection was 10 µL, and the overall analysis run timeframe was 6 min at a flow velocity of mobile phase at 1 mL in a min.

Preparation of LVME and TCBE solutions

Stock LVME and TCBE solutions having quantities 375 µg/mL LVME and 500 µg/mL TCBE are prepared by dissolving LVME (37.5 mg) and TCBE (50 mg) compounds in mobile phase (100 mL). Working LVME and TCBE solutions were made by mixing appropriate volume size of stock LVME and TCBE solution (375 µg/mL LVME and 500 µg/mL TCBE) with mobile phase to concentrations of 37.5 µg/mL LVME and 50.0 µg/mL TCBE.

Suspension sample

In 100 mL of mobile phase, a portion of Clobend-L suspension (1 mL), corresponding to 37.5 mg LVME and 50 mg TCBE, was dissolved. The suspension was subsequently 30 min sonicated and after that filtered. This is stock LVME and TCBE Clobend-L suspension solution having quantities 375 µg/mL of LVME and 500 µg/mL of TCBE. Working Clobend-L suspension LVME and TCBE solutions for investigation were made by mixing appropriate volume size of stock LVME and TCBE solution (375 µg/mL LVME and 500 µg/mL TCBE) with mobile phase to have concentrations of 37.5 µg/mL LVME and 50.0 µg/mL TCBE.

Linearity curves

Linearity was revealed from 18.75 µg/mL to 56.25 µg/mL and 25 µg/mL to 75 µg/mL sample concentrations expending five calibration levels (LVME – 18.75 µg/mL, 28.13 µg/mL, 37.50 µg/mL, 46.875 µg/mL and 56.25 µg/mL; TCBE – 25 µg/mL, 37.5 µg/mL, 50 µg/mL, 62.5 µg/mL and 75 µg/mL) for LVME and TCBE, respectively. The linear regression approach was employed to evaluate the data. The peak area of LVME/TCBE was plotted against concentrations of LVME/TCBE. Linearity was explained using a linear expression of regression, and the correlation coefficient also being calculated as well.

Content assay of LVME and TCBE in Clobend-L suspension

Working Clobend-L suspension LVME and TCBE solutions made in section "Suspension sample" was assessed adopting conditions in section "Chromatography assaying conditions". The content of LVME and TCBE in Clobend-L suspension were evaluated using either LVME/TCBE linearity curves or LVME/TCBE linear expression of regression.

Degradation studies

The Clobend-L suspension LVME and TCBE solution (375 µg/mL of LVME and 500 µg/mL of TCBE) was lay open to stress conditions comprising alkali caused hydrolysis, acid caused hydrolysis, peroxide caused oxidation, dry heat caused degradation and light caused degradation[11].

Alkali/acid/peroxide caused degradation

Accurately measured (10 mL) Clobend-L suspension LVME and TCBE solution (375 µg/mL of LVME and 500 µg/mL of TCBE) was mixed with either 0.1N NaOH (10 mL, alkali caused hydrolysis)/ 0.1 N HCl (10 mL, acid caused hydrolysis)/ 30% peroxide (10 mL, peroxide caused oxidation). The obtained solutions were then sonicated for 30 min at ambient temperatures in a dark area before being diluted to a volume size of 100 mL using mobile phase. These samples were assessed for LVME and TCBE adopting conditions in section "Chromatography assaying conditions".

Dry heat/light caused degradation:

Accurately measured (10 mL) Clobend-L suspension LVME and TCBE solution (375 µg/mL of LVME and 500 µg/mL of TCBE) was made exposed to dry hear (60 ^{o}C for 30 min, dry heat caused degradation)/ sun light (6 hr, light caused degradation). The obtained solutions were then being diluted to a volume size of 100 mL using mobile phase. These samples were assessed for LVME and TCBE adopting conditions in section "Chromatography assaying conditions".

3. Results and discussion

Chromatography LVME and TCBE assaying conditions optimization

A succession of solvent combinations (Na_2HPO_4: Methanol and Na_2SO_4: Methanol), ratio of Na_2HPO_4 and Na_2SO_4 solutions and diverse C18 chromatography column categories (Kromasil, Aligent, Waters and ACE) were studied to confirm good resolution, peak shapes and apt retention times of LVME and TCBE. The HPLC LVME and TCBE chromatogram was displayed in Figure 2. The best result was achieved by comparing the peak shapes and resolutions of the LVME and TCBE drugs at a pH of 3.5 and methanol-0.1M Na_2SO_4 (40-60; v/v) with flow measure of 1.0 mL/min.

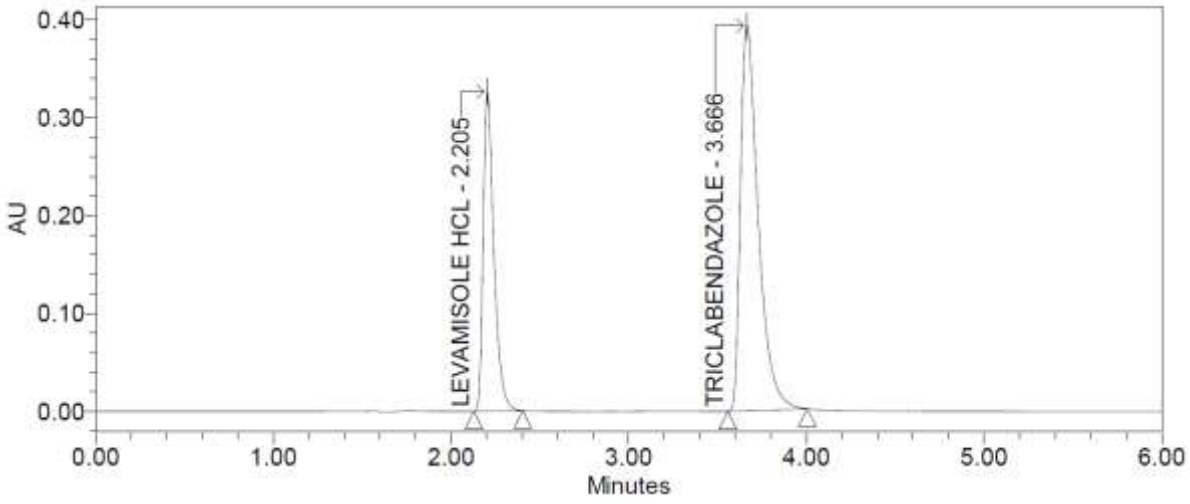

Figure: 2 LVME and TCBE chromatogram

Method validation

Validation of developed LVME and TCBE combination stability indicating assaying approach was completed as specified by ICH recommendations[12].

Linearity

LVME and TCBE calibration curves encompassed five calibration standards comprising the concentration range 18.75 µg/mL to 56.25 µg/mL and 25 µg/mL to 75 µg/mL, respectively. The peak areas of LVME and TCBE was charted towards the quantity of LVME and TCBE to create their individual calibration curve. The slope, intercept, and R^2 for LVME and TCBE were measured applying linear regression, and the values are displayed below.

LVME linear calibration equation: LVME peak area = 36173 x + 6134.7

LVME R^2 score = 0.9999

TCBE linear calibration equation: TCBE peak area = 55342 x - 26332

TCBE R^2 score = 1.0000

The R^2 score was >0.9990, indicating that the dataset was well-fitting to the regression line.

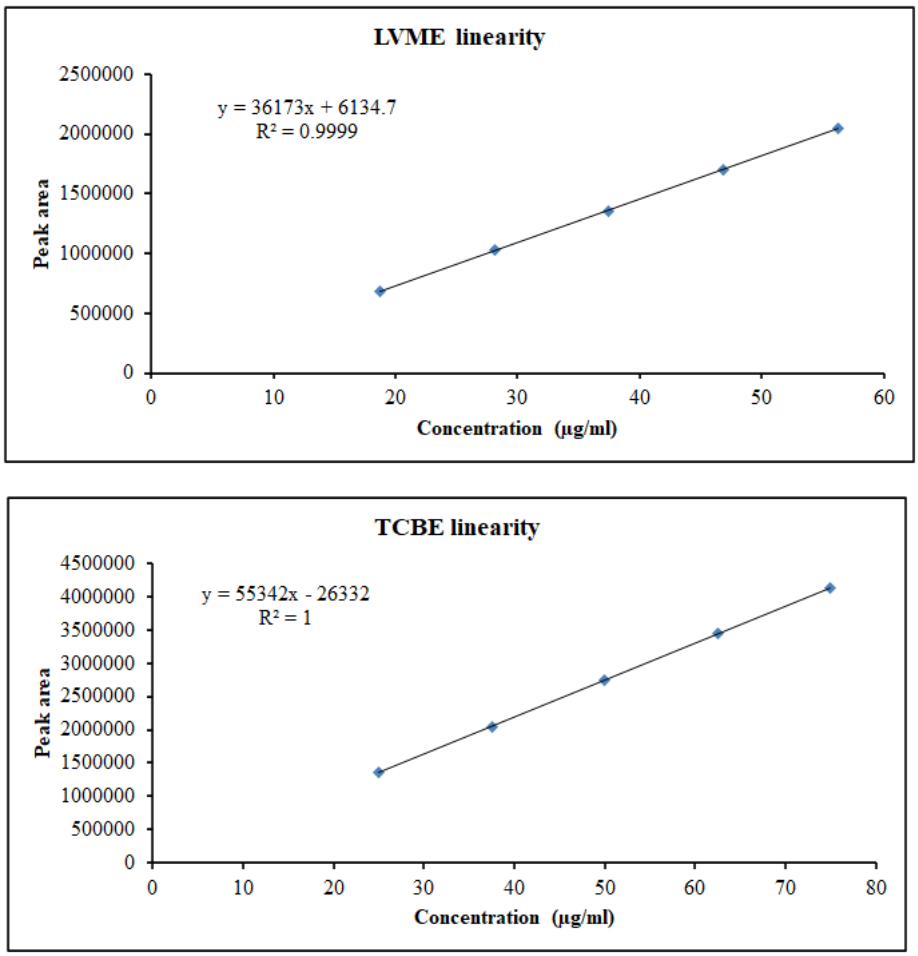

Figure: 3 LVME and TCBE linearity figures

Precision and accuracy

Six standard LVME and TCBE solutions (37.5 µg/mL of LVME and 50.0 µg/mL of TCBE) were used to ascertain the accuracy besides precision. These six samples were analysed adopting conditions in section "Chromatography assaying conditions" in a single day. Table 1 displays the outcomes. The statistics in Table 1 signalled that the accuracy (% recovery: 98.63% for LVME and 98.99% for TCBE) and precision (%RSD: 0.215% & 0.213% for LVME and 0.164 & 0.165% for TCBE) met the tolerance criteria.

Table: 1 Accuracy and precision for LVME and TCBE

Injection sample	LVME precision (peak area)	TCBE precision (peak area)	LVME accuracy (%recovery)	TCBE accuracy (%recovery)
1	1363916	2741916	98.95	99.13
2	1360763	2737295	98.72	98.96
3	1358494	2742405	98.56	99.14
4	1355562	2740914	98.35	99.09
5	1360374	2731005	98.69	98.73
6	1357438	2735142	98.48	98.88
Mean	1359425	2738113	98.63	98.99
SD	2918.822	4488.860	0.210	0.163
RSD	0.215	0.164	0.213	0.165

LOD and LOQ

The working LVME and TCBE solution (37.5 µg/mL LVME and 50.0 µg/mL TCBE) was subsequently diluted and injected into 25 cm × 0.46 cm, 5 µm, column Kromasil RP-C18 to establish signal-to-noise (STN) ratios of 3:1 (for establishing LOD) and 10:1 (for establishing LOQ). Empower two software was applied to compute the STN ratios for LVME and TCBE. The LVME detection and quantitation limits were determined as 0.035 µg/mL with STN of 3.9 and 0.116 µg/mL with STN of 10.2 (Figure 4). The TCBE detection and quantitation limits were determined as 0.038 µg/mL with STN of 3.2 and 0.128 µg/mL with STN of 10.8 (Figure 4).

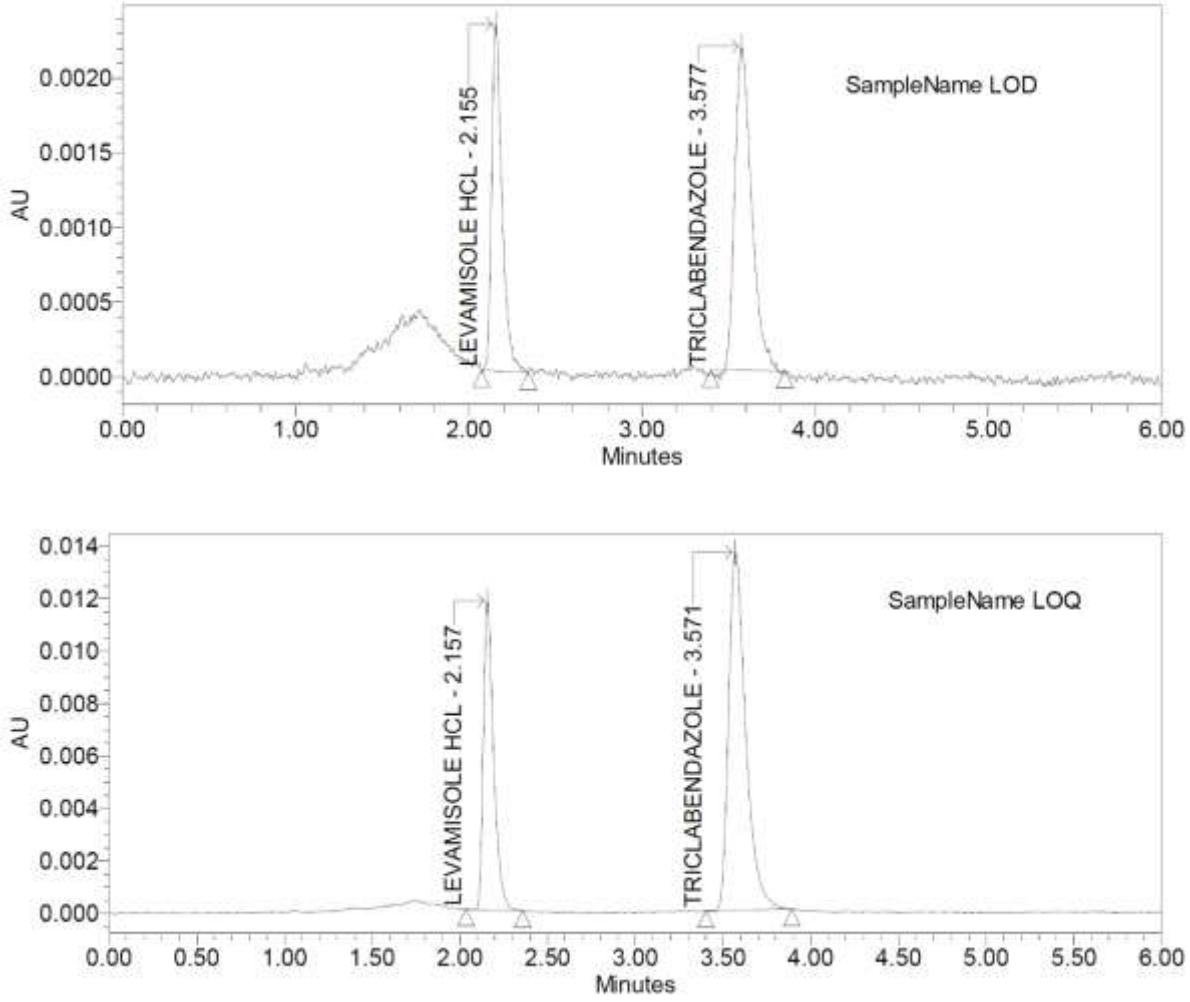

Figure: 4 Chromatograms for LVME and TCBE at LOD and LOQ range quantity

Recovery/selectivity

The average recoveries of LVME and TCBE is evaluated by analysing spiked Clobend-L suspension LVME and TCBE samples at three unique levels of LVME and TCBE (three replicates each level), which include 18.563 µg/mL, 37.125 µg/mL and 55.688 µg/mL for LVME and 24.750 µg/mL, 49.50 µg/mL and 74.250 µg/mL for TCBE. The values in Table 2 signalled that the %recovery (99.64% to 100.77% for LVME and 99.26% to 100.31% for TCBE) met the tolerance criteria and also signifies selectivity due to the absence of excipient interference.

Table: 2 Selectivity/recovery fallouts for estimating LVME and TCBE in Clobend-L suspension

LVME				TCBE			
µg/mL		Percentile		µg/mL		Percentile	
Added	**Found**	**Recovery**	**Mean**	**Added**	**Found**	**Recovery**	**Mean**
18.563	18.66	100.50	100.52	24.750	24.66	99.62	99.26
18.563	18.68	100.64		24.750	24.47	98.88	
18.563	18.64	100.42		24.750	24.57	99.27	
37.125	37.15	100.06	99.64	49.500	49.49	99.99	100.03
37.125	36.79	99.09		49.500	49.52	100.03	
37.125	37.04	99.77		49.500	49.54	100.07	
55.688	56.28	101.06	100.77	74.250	74.56	100.41	100.31
55.688	56.04	100.63		74.250	74.49	100.32	
55.688	56.03	100.62		74.250	74.39	100.19	

Robustness

The impact of purposeful alterations in the LVME and TCBE assessment conditions, namely: mobile phase methanol volume ratio (± 5%); pH in mobile phase buffer (± 0.1); temperature (± 5°C); detection nanometers (± 2) and mobile phase flow stream (± 0.1) was studied. Standard LVME and TCBE solutions (37.5 µg/mL of LVME and 50.0 µg/mL of TCBE) were used to assess each condition, and the percent RSD relating to the area of the normal versus modified situations were calculated. The statistics in Table 3 signalled that the robustness met the tolerance criteria.

Table: 3 Robustness fallouts for LVME and TCBE combination assaying methodology

Alternation in	Peak area*	RSD	SD
LVME			
Methanol composition	1363704.0	1.4	19656.5
Flow rate	1359767.3	1.9	25899.1
Detection nm	1361885.3	1.7	23396.0
pH	1376464.2	0.7	10297.9
Temperature	1339550.3	1.6	22056.2
TCBE			
Methanol composition	2729474.7	1.3	35227.4
Flow rate	2733588.3	1.8	48745.4
Detection nm	2741432.3	1.8	48685.5
pH	2754738.2	0.6	17301.1
Temperature	2736130.7	1.3	34898.2

* mean – three determinations

Degradation

The analysis of Clobend-L suspension LVME and TCBE solution (375 µg/mL of LVME and 500 µg/mL of TCBE) exposed to alkali caused hydrolysis, acid caused hydrolysis, peroxide caused oxidation, dry heat caused degradation and light caused degradation to assess the specificity/stability indicating feature of the LVME and TCBE analysing method indicated the occurrence of degradation products. The percentage stability and instability of LVME and TCBE were provided in Table 4. The peak purity testing outcomes for LVME and TCBE during this study were also provided in Table 4. In the retention time of LVME and TCBE, however, no extra signals were detected (Figure 5). It was concluded that there was none interference in the enumeration and identification of LVME and TCBE, and that the produced signal was owing only to LVME and TCBE.

Table: 4 LVME and TCBE degradation study fallouts

Condition	LVME				TCBE			
	% Stability	% Instability	Purity angle	Purity threshold	% Stability	% Instability	Purity angle	Purity threshold
1	90.49	9.51	0.191	0.467	91.91	8.09	0.290	0.588
2	93.07	6.93	0.101	0.567	92.64	7.36	0.288	0.487
3	94.74	5.26	0.380	0.665	93.67	6.33	0.290	0.586
4	89.84	10.16	0.376	0.566	90.51	9.49	0.291	0.687
5	91.05	8.95	0.272	0.766	92.73	7.27	0.391	0.587

1. Acid caused hydrolysis; 2. Alkali caused hydrolysis; 3. Peroxide caused oxidation; 4. Dry heat caused degradation; 5. Light caused degradation

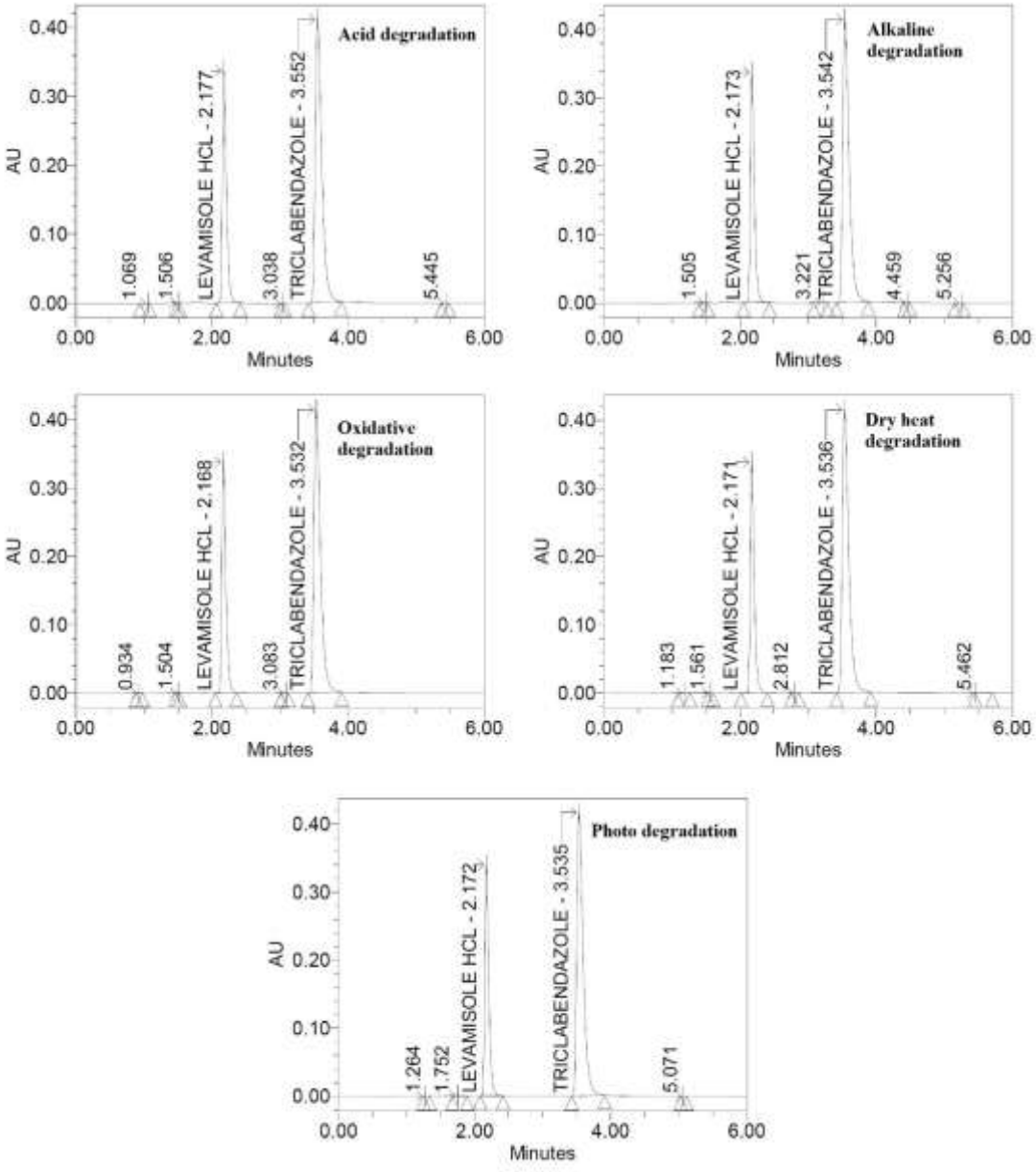

Figure: 5 LVME and TCBE degradation study chromatograms

4. Conclusion

For the quantification of LVME and TCBE, a "stability indicating HPLC technique" was created and validated. This method can be successfully applied for LVME and TCBE combination analysis as well as for LVME and TCBE stability study. On the whole, the "stability indicating HPLC technique" provides prominent through put technique for combo determination of LVME and TCBE in Clobend-L suspension and bulk with excellent sensitivity, precision, selectivity, and accuracy.

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