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Reverse phase high-performance liquid chromatographic method development for simultaneous determination of biologically active and thermally stable 1,1'-bis(3-methyl-4-hydroxyphenyl)cyclohexane and 1,1'-bis(3,5-dibromo-4-hydrophenyl)cyclohexane

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ABSTRACT

A rapid and sensitive reverse phase high-performance liquid chromatographic (RP-HPLC) method has been developed and validated successfully for the simultaneous determination of biologically active and thermally stable 1,1'-bis(3-methyl-4-hydroxyphenyl) cyclohexane (MEBC) and 1,1'-bis(3,5-dibromo-4-hydrophenyl)cyclohexane (TBBC). Separation of analytes were achieved within 10min by using Phenomenex Luna C18(2) (25mm x 4.26mm, 5 µm) column. The method has excellent linearity (R^2 > 0.9990), precise (RSD < 2), accurate (recovery of 99.43-99.76 % for MEBC and 99.69-100.12 % for TBBC, specific and robust. Limit of detection and limit of quantitation were 1.5 µg $mL^{-1}$and 4.0µg mL^{-1}, respectively. The developed method may be useful bioavailability and bioequivalence studies.

Keywords: bisphenol, RP-HPLC, photo diode array, thermal study

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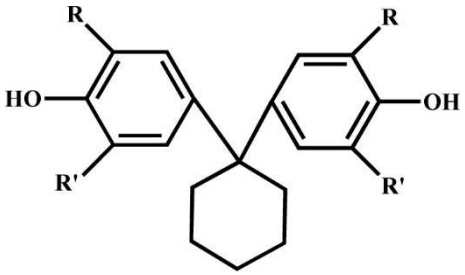
INTRODUCTION

Bisphenols are used for a class of chemical compounds bearing two hydroxyphenyl moieties connected via a carbon or sulfur bridge [1]. For many decades, they are widely used in the manufacturing of epoxy resins and polycarbonates [2].Bisphenols are also used in the synthesis of advanced high temperature composite materials used in the electronic and aerospace industries [3]. Previous studies have indicated that these chemicals can be released into the environment during manufacturing processes and through leaching from consumer products or polymer matrices after incorporation with important implications for human exposure. Recently, concerns about these chemicals were exacerbated by toxicity studies showing various adverse effects on human and animals [4, 5]. Bisphenols like bisphenol A, bisphenol AF and bisphenol E in the environment have attracted attention because they are endocrine disruptors [6-9]. Because of health concerns of these bisphenols especially bisphenol A have been replaced by several chemicals that are structurally similar with two hydroxyphenyl functionalities [10]. Recent studies have been reported that some of bisphenol derivatives showsantioxidant, antiviral activity, antagonistic activity for an estrogen receptor and act as a selective estrogen receptor modulator [11-15]. Because of wide spread applications importance and environmental aspects of these bisphenols, simple and robust test methods are needed to determine the presence and amount of bisphenols. To our knowledge no work has been reported on method development and validation of bisphenol-C derivatives, which encouraged us to taken up present work.

In this paper, we had developed a simultaneous determination of biologically active and thermallystable 1,1'-bis(3-methyl-4-hydroxyphenyl)cyclohexane (MEBC) and 1,1'-bis(3,5-dibromo-4-hydrophenyl)cyclohexane (TBBC). General molecular structure is presented in Fig 1.



MEBC : R = CH_3 and R' = H
TBBC : R = R' = Br

Fig 1. Molecular structure of MEBC and TBBC

MATERIALS AND METHODS

Experimental

High purity MEBC and TBBPC were synthesized and crystallized according to our recent publication [16] and further purified by column chromatography. HPLC grade acetonitrile (ACN), methanol (MeOH) and other solvents were purchased from Spectrochem Pvt. Ltd, Mumbai (India).Analytical grade hydrochloric acid, sodium hydroxide pellets and hydrogen peroxide solution 30 %(v/v) were obtained from Ranbaxy Fine Chemicals, New Delhi (India). Water used in all experiments was double distilled and purified by a Milli-Q system (Millipore, Milford, MA, USA).

Instrumentation and Chromatographic Conditions

Development and validation of methodwas performed on a Shimadzu LC-10A HPLC system (Shimadzu, Kyoto, Japan) consisting of two LC-10Advp pumps, SPD-M10Avp photo-diode array detector and a Rheodyne manual injector model 7725i with 20µL loop (Shimadzu, Kyoto, Japan). The data were acquired and processed with LC solution software (Shimadzu).

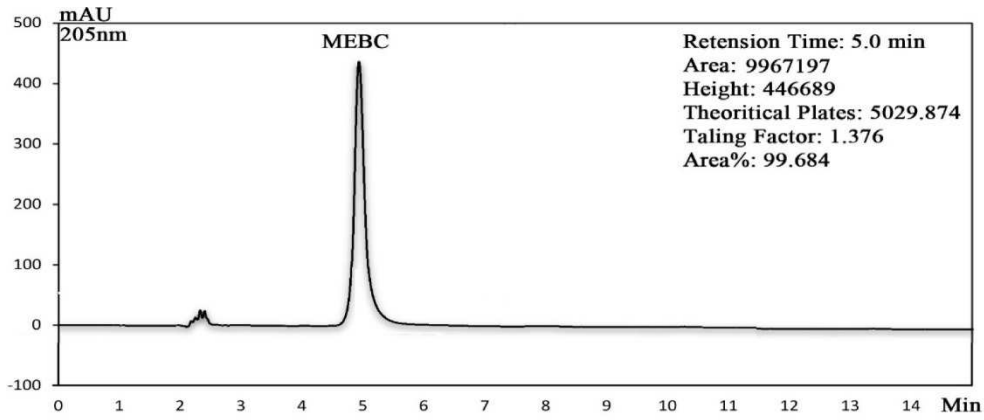


Fig 2. Standard chromatogram of MEBC

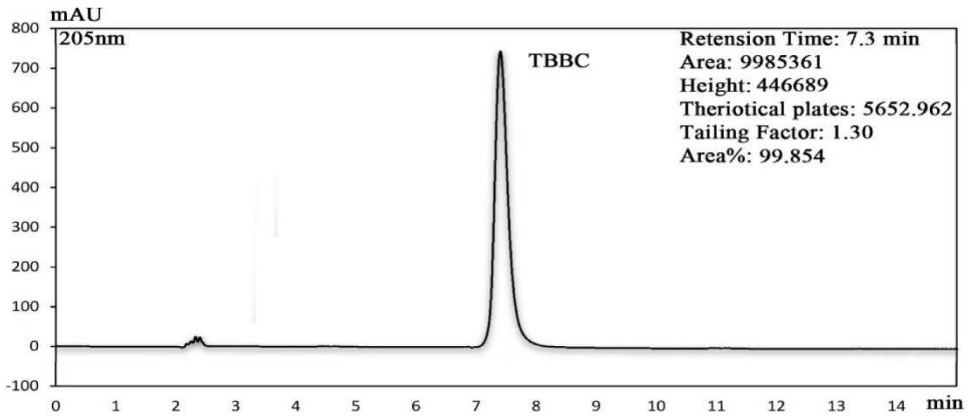


Fig 3. Standard chromatogram of TBBC

The analysis was performed on a Phenomenex Luna C18 (2) (250 mm x 4.26mm, 5 µm) column. The mobile phase was acetonitrile: 0.1% aq. acetic acid (70: 30, v/v). Mobile phase was filtered through 0.45 and 0.22 µm teflon filter prior to its use. The injection volume was 20 µL. The flow rate of the mobile phase was 1.0 mL min^{-1}. The HPLC measurements were carried out at 25± 1 ^{o}C. Detection was performed at 205nm.

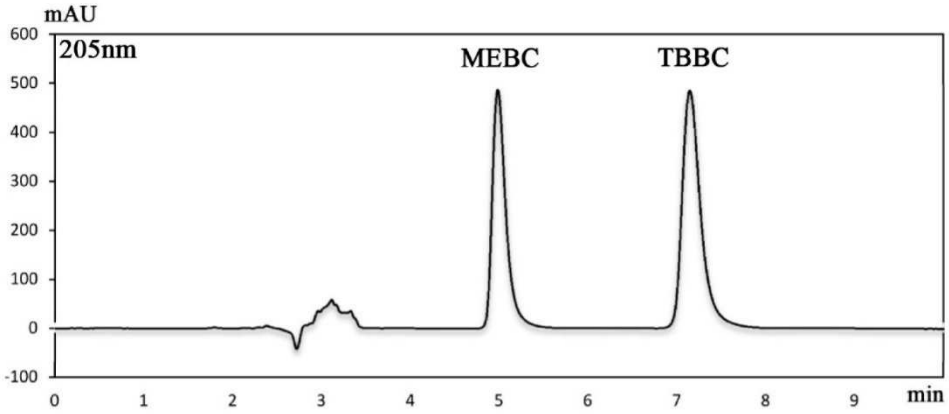


Fig 4. Chromatogram of standard preparation

Preparation of stock solutions

Stock solution of (500 µg mL^{-1}) of MEBC and TBBC were prepared in 25 mL methanol and sonicated for 2 min. From this solutions each of 25 mL solutions were transferred to 50 mL volumetric flasks. MEBC, TBBC and their mixture were filtered through a 0.45 and 0.22 µm teflon filters.

Preparation of standard solutions

A 10 mL(50 µg mL^{-1})standard solution of each of MEBC and TBBC was prepared and stored at room temperature.

Preparation of test solutions

A series of test solutions ranging from 10-90µg mL^{-1} were prepared for calibration purpose. A 10 mL of each of 50%, 100% and 150% solutions were prepared for accuracy purposes and stored at room temperature.

Method Validation

HPLC method validation was carried out by considering various parameters like accuracy, precision, linearity, robustness, degradation, limit of detection (LOD), and limit of quantification (LOQ) as per ICH guidelines Q2A and Q2B [17, 18].

Precision

The precision of the method was checked by analysing the standard solution at least five times. In order to check method precision, six different sets of solutions having (50 µg mL^{-1}) concentration were prepared for intra- and inter day precision. Each of the solutions were injected in duplicate.

Linearity

The linearity of the method was checked by using a series of solutions (10-90µg mL^{-1} or 20-180%) injected in duplicate and average area was considered for the least squares analysis.

Accuracy

The accuracy of the method was assessed by recovery of 50, 100 and 150 % solutions. For each of the solutions, the measurements were carried out in duplicate and average areas were considered.

Robustness

The robustness of the method was checked by assaying standard solutions under different analytical conditions deliberately changed from the original one. For each different analytical conditions the standard solutions were prepared separately and assayed. The results of the assay were not affected by varying the analytical conditions conforming excellent agreement.

Solution stability

The stability of the solution of standard solutions was assayed at two different temperatures namely 5°C and 25± 1 ^{o}C without protection of light and assayed at the interval of 12h (up to 48 h). The resultant data were compared with freshly prepared solution.

Degradation

The degradation assay was performed in acidic, alkaline, oxidizing, thermal and photolytic conditions. Acidic degradation was performed by mixing 1 mL of standard solution and 1N HCl solution, mixed well and kept at room temperature for 4 h and then mixture was neutralized and diluted to 10 mL. Similarly degradation under alkaline and oxidizing conditions was performed by using 1N NaOH and 30% v/v H_2O_2(v/v). For thermal degradation assay, the solution was heated at 60 ^oC for 4 h and cooled to room temperature. All the samples were further assayed for their stability.

RESULTS AND DISCUSSION

Figs 2 and 3 shows chromatograms of MEBC and TBBC, respectively. From the chromatograms, it is observed that MEBC and TBBC showed 99.7 and 99.8% purity. Similarly Fig4 shows chromatogram of the equivi mixture of MEBC and TBBC.

Precision

Precision data of MEBC and TBBC are presented in Table 1. It is observed that mean and % RSD values for intra and inter day assay are 100.30, 0.16 % and 99.67, 0.31 % and 99.78, 0.27 %and 99.87, 0.26 %, respectively, for MEBC and TBBC. Intermediate precision was established by determining the overall intra-day and inter-day method precision for the assay. For MEBC, overall intermediate assay (n=12) was 99.89 and 0.19 % RSD. For TBBC, overall intermediate precision was 99.83 and 0.06 % RSD. From the observed fact it is concluded that the method precision is excellent.

Table 1: Precision data of MEBC and TBBC

Set	MEBC (% Assay)		TBBC (% Assay)	
	Intraday (n=6)	Interday (n=6)	Intraday (n=6)	Interday (n=6)
1	99.82	100.01	100.02	99.96
2	100.04	100.06	99.67	99.90
3	100.30	99.70	99.88	100.09
4	99.96	99.88	99.47	99.58
5	100.07	99.67	100.12	99.53
6	99.99	99.20	99.53	100.17
Mean	100.03	99.76	99.78	99.87
Standard deviation	0.16	0.31	0.27	0.26
% RSD	0.16	0.31	0.27	0.26
Overall intermediate precision				
Mean	99.89		99.83	
Standard deviation	0.19		0.06	
% RSD	0.19		0.06	

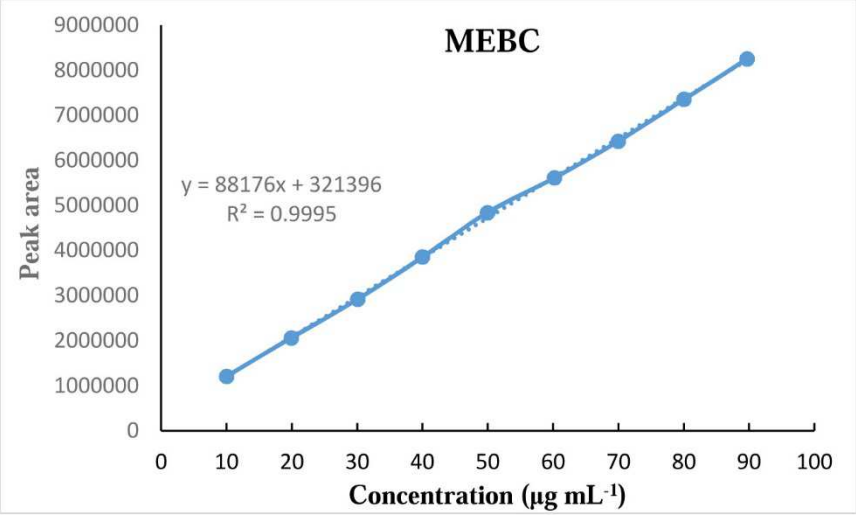


Fig 5 Linearity curve of MEBC

Linearity

The linearity of the method was assayed by assaying different standard solutions (10-90µg mL^{-1}) of MEBC and TBBC. The calibration curves were constructed by least squares fitting of the experimental data (Table 2). The

calibration plots for MEBC and TBBC are presented in Figs 5 and 6, respectively. The least squares equations and regression coefficients for MEBC and TBBC are as under. For MEBC, Peak are = 88176 C + 321396 and R^2 = 0.9995. For TBBC, Peak area = 105086 C + 568118 and R^2 = 0.9992. The least squares data of MEBC and TBBC showed excellent linearity of the method.

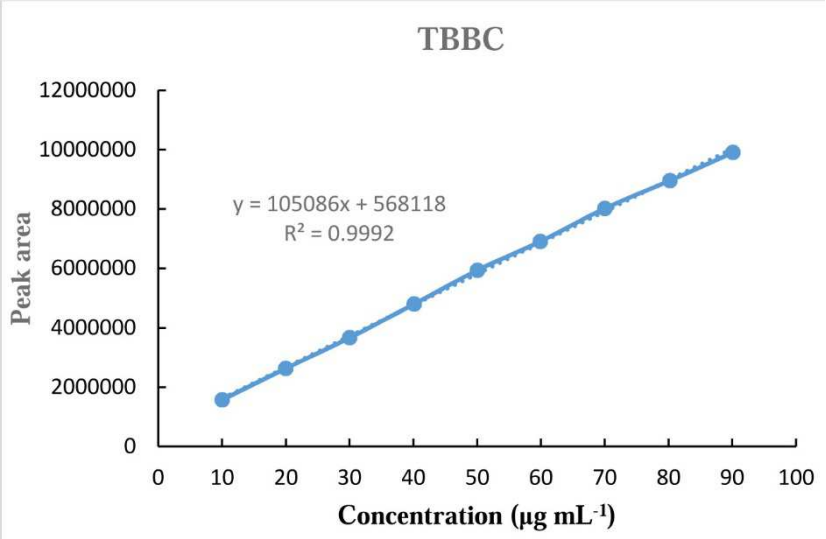


Fig 6 Linearity curve of TBBC

Table 2 Linearity data for MEBC and TBBC

Concentration (µg mL^{-1})	Mean Area MEBC, AU	Mean Area TBBC, AU
10.00	1209996	1577626
20.00	2065657	2633239
30.00	2919413	3666733
40.00	3857006	4801961
50.00	4844072	5940897
60.00	5614398	6915604
70.00	6426793	8025469
80.00	7355322	8958229
90.00	8255879	9907925

Accuracy

The accuracy assay of the method was performed by assaying three different concentration levels (50, 100 and 150%)and the data are reported in Tables 3 and 4, respectively for MEBC and TBBC. The observed amounts and % recovery of the samples were determined according to following relationships.

$$\text{Amount found} = \frac{\text{Area of sample}}{\text{Area of standared sample}} \times \text{concentration of standarad sample}$$

$$\%\ \text{Recovery} = \frac{\text{Amount found}}{\text{Amount taken}} \times 100$$

Table 3 Recovery data for MEBC

% Level	Obs. No.	Mean Area, AU	Amount Taken (µg mL^{-1})	Amount found, (µg mL^{-1})	% Recovery	Mean % recovery	Standard deviation	%RSD
50	1	2381704	25.00	24.91	99.65	99.76	0.23	0.23
	2	2388694	24.98	24.99	100.02			
	3	2380755	25.00	24.90	99.61			
100	1	4757538	50.08	49.76	99.37	99.46	0.21	0.21
	2	4762277	49.96	49.81	99.70			
	3	4747859	50.00	49.66	99.32			
150	1	7130582	74.88	74.58	99.60	99.43	0.37	0.37
	2	7082091	74.82	74.08	99.01			
	3	7097866	75.00	74.24	99.69			

Table 4 Recovery data for TBBC

% Level	Obs. No.	Mean Area, AU	Amount taken (µg mL^{-1})	Amount found (µg mL^{-1})	% Recovery	Mean % recovery	Standard deviation	%RSD
50	**1**	2956352	25.00	24.99	99.96	99.69	0.39	0.40
	2	2932571	24.98	24.79	99.24			
	3	2956048	25.02	24.99	99.87			
100	**1**	5889387	49.92	49.78	99.73	99.89	0.18	0.18
	2	5906029	50.00	49.92	99.85			
	3	5915096	49.96	50.00	100.08			
150	**1**	8937216	74.88	75.55	100.89	100.12	0.68	0.68
	2	8834247	74.76	74.68	99.89			
	3	8828409	74.94	74.63	99.58			

Robustness

The robustness of the method was assayed under experimental conditions, such as the flow rate (±0.1 ml min^{-1}), mobile phase composition (Water-Acetonitrile, 29:71 and 31:69, v/v) and different column temperature. The robustness data of MEBC and TBBC are presented in Tables 5 and 6, respectively. From Tables 5 and 6, it is clear that % assay did not change appreciably in spite change in analytical conditions. System suitability parameters (theoretical plates and asymmetry) are also found almost constant confirming excellent robustness of the method.

Table 5 Robustness assay of MEBC

Robust condition		% Assay	System suitability parameters	
	ACN:H_2O		Theoretical plates	Asymmetry
Mobile phase	70:30	99.80	4965	1.32
Column temperature, ^oC	25	99.80	5015	1.30
Flow rate, mL min^{-1}	1	99.72	4981	1.29
Mobile phase	69:31	100.21	5154	1.31
Column temperature, ^oC	30	99.57	4801	1.34
Flow rate, mL min^{-1}	0.9	99.29	4906	1.35
Mobile phase: ACN: Water	71:29	100.41	4993	1.36
Column Temperature, ^oC	35	99.83	4864	1.32
Flow rate, mL min^{-1}	1.1	99.22	4858	1.33

Table 6 Robustness assay of TBBC

Robust condition		% Assay	System suitability parameters	
	ACN:H_2O		Theoretical plates	Asymmetry
Mobile phase	70:30	100.31	5817	1.30
Column temperature, ^oC	25	100.18	5814	1.29
Flow rate, mL min^{-1}	1	99.32	5808	1.30
Mobile phase	69:31	99.86	6094	1.31
Column Temperature, ^oC	30	99.42	5670	1.31
Flow rate, mL min^{-1}	0.9	99.98	5663	1.32
Mobile phase	71:29	99.49	5879	1.32
Column Temperature, ^oC	35	100.61	5696	1.29
Flow rate, mL min^{-1}	1.1	100.49	5858	1.31

Solution Stability

The stability of standard solution was assayed at 5^{o}C and 25^{o}C at the interval of 12h and data are reported in Table 7. From Table 7, it is clear that solution stability is found excellent.

Table 7 Stability data of MEBC and TBBC

Interval, h	% Assay for test at 25°C			
	MEBC	**% Difference**	**TBBC**	**% Difference**
Initial	99.85	-	99.96	-
12	99.62	0.23	99.49	0.47
24	99.63	0.22	99.72	0.24
36	99.56	0.29	99.93	0.03
48	99.65	0.20	99.30	0.66
	% Assay for test at 5 °C			
	MEBC	**% Difference**	**TBBC**	**% Difference**
Initial	99.89	-	99.78	-
12	99.88	0.01	99.49	0.29
24	99.01	0.88	99.68	0.10
36	99.33	0.56	99.57	0.21
48	99.73	0.16	99.60	0.18

LOD & LOQ

Limits of detection (LOD) and quantification were determined by determining signal to noise ratios. For LOD and LOQ, 1.5 µg $mL^{-1}$and 4.0 µg mL^{-1}solutions were injected and corresponding chromatograms are presented in Figs. 8 and 9, respectively. Theoretical values of LOD and LOQ should be 3 times and more than 10 times of blank sample. Observed LOD values for MEBC and TBBC are 3.1 and 3.9, respectively. Similarly, observed LOQ values of MEBC and TBBC are 10.3 and 11.0, respectively. Thus, LOD and LOQ values are some water better than expected

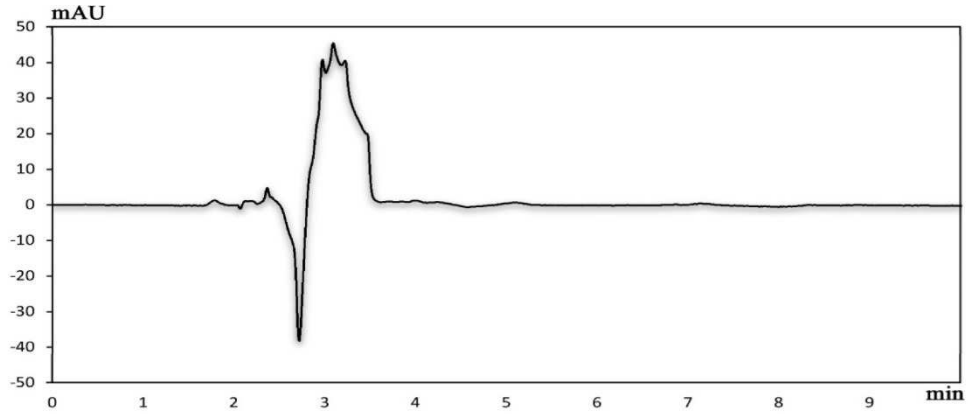


Fig 7 Chromatogram of blank sample

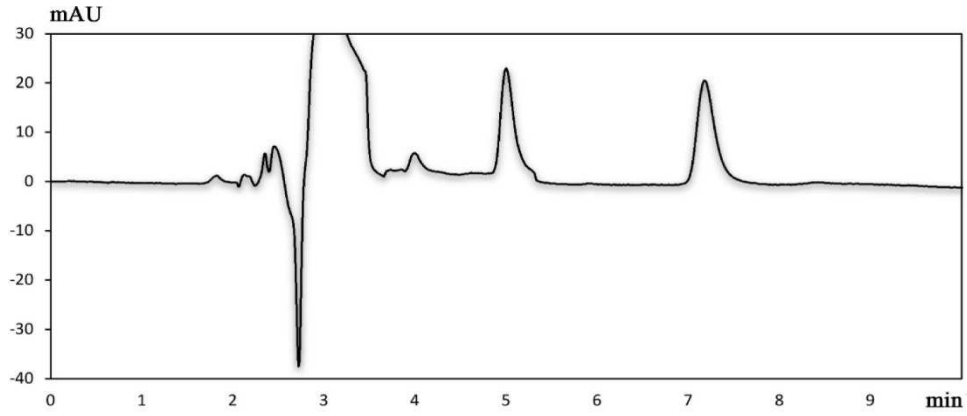


Fig 8 Chromatograms of LOD assay of MEBC and TBBC

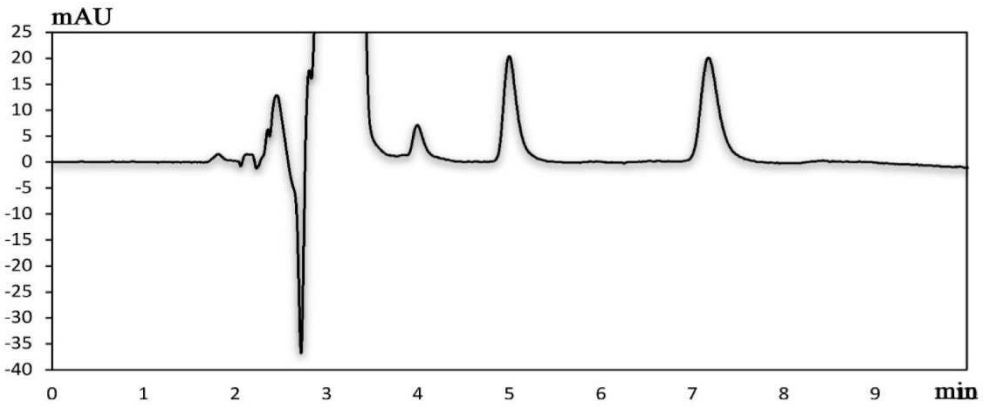


Fig 9 Chromatograms of LOQ assay of MEBC and TBBC

Degradation assay

Figs.10 to 13 show chromatograms after degradation assay. The % degradation products and under different conditions are reported in Table 8 from which it is observed that both the analytes degraded up to about 0.06-0.92% confirming excellent resistant to degradation under selected conditions.

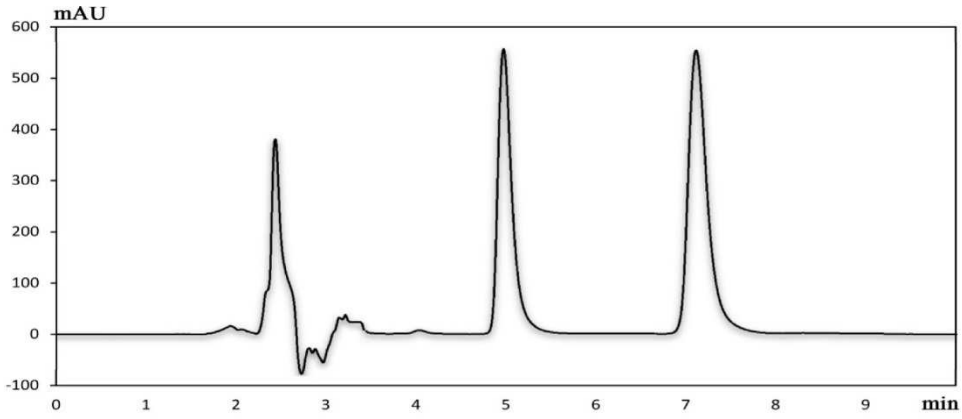


Fig 10 Chromatograms of MEBC and TBBC after acid degradation

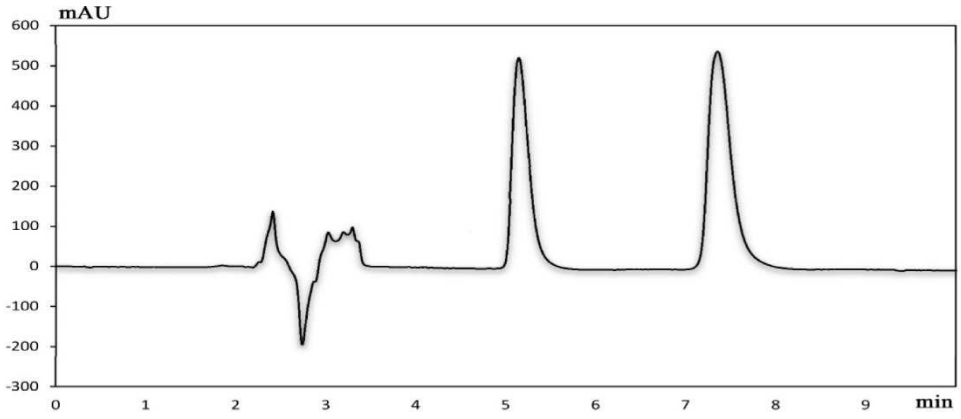


Fig 11 Chromatograms of MEBC and TBBC after alkali degradation

Table 8% Degradation of analytes under different degradation conditions

Conditions	*% Degradation*	
	MEBC	*TBBC*
Acidic	0.5	0.22
Alkaline	0.73	0.43
Oxidative	0.92	0.84
Thermal	0.06	0.07

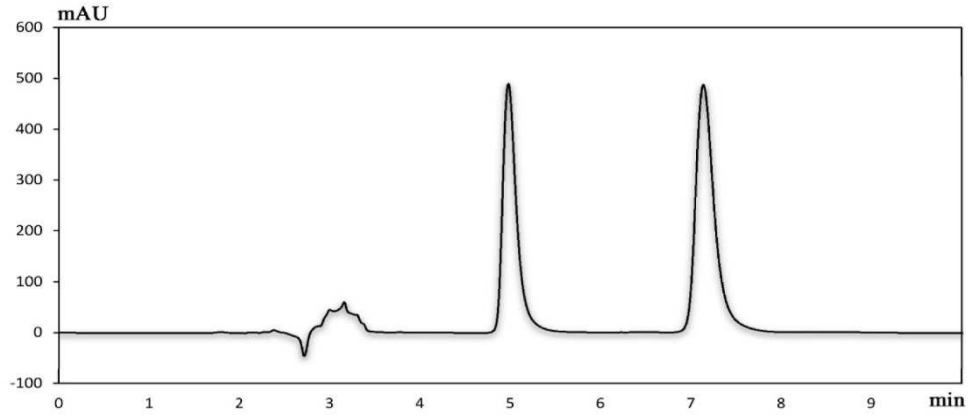


Fig 12 Chromatograms of MEBC and TBBC after oxidative degradation

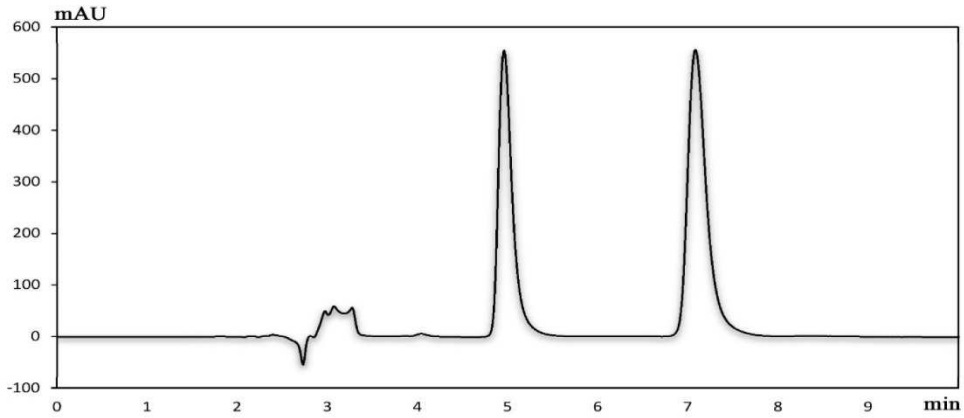


Fig 13 Chromatograms of MEBC and TBBC after thermal degradation

CONCLUSION

The method is developed and validated successfully for MEBC and TBBC using RP-HPLC. The developed method is simple, faster, sensitive, specific and reproducible. It can be used in bioavailability and bioequivalence study.

Acknowledgements
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