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A Stability Indicating RP-HPLC Method for Simultaneous Estimation of Ethylhexyl Triazone and Bemotrizinol in Sun Care Formulations

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ABSTRACT

A novel stability indicating RP-HPLC method was developed for simultaneous estimation of Ethylhexyl triazone and Bemotrizinol in sun care formulations. Separation of Ethylhexyl triazone, Bemotrizinol and their degradation products was achieved on Waters symmetry C18 (150 × 3.9 mm, 5µ) as stationary phase and mixture of pH 4.50 ammonium formate buffer and 1,4-dioxane (20:80, v/v) as mobile phase. Both analytes were simultaneously measured at UV detection of 311 nm. Method was validated for specificity, method precision (Intra-day and inter-day), linearity, accuracy and robustness as per ICH guideline. Ethylhexyl triazone and Bemotrizinol were linear in the concentration range of 0.10-225 µg/mL and 0.12-225 µg/mL respectively with correlation coefficient of > 0.999. This method can be utilized in cosmetic industries to monitor both analytes in sun care formulations to assure the quality of the products.

Keywords: Ethylhexyl triazone, Bemotrizinol, Stability indicating

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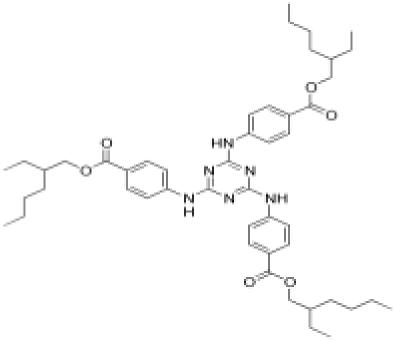
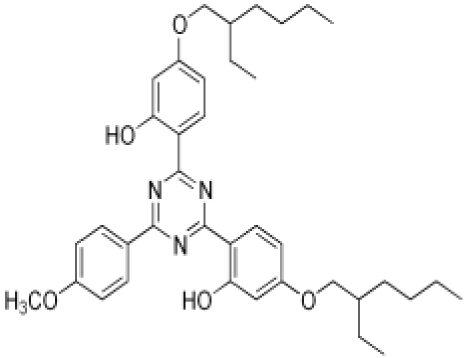
INTRODUCTION

Radiations emitted by the sun and more particularly by the ultraviolet (UV) rays provoke harmful effects on the skin. The use of sunscreen (Sun care) product that absorbs or reflects some of the sun’s ultraviolet (UV) radiation on the skin exposed to sunlight and thus help to prevent or minimize these harmful effects on human health. The use of sunscreens against UV B (290-320 nm) and UV A (320-400 nm) has led the cosmetic industry to develop formulations containing many ultraviolet organic sunscreens in association with chemical structures that is more complex[1].

Ethylhexyl triazone (ETZ) and Bemotrizinol (BT) are organic sunscreens used in sun care formulations to provide high SPF (Sun protection factor) value and give excellent protection over UVA and UVB radiation. ETZ is an oil soluble UVB filter and has an absorption maximum of 314 nm. Due to its insolubility in water and affinity to the skin keratin, it is particularly suitable for water-resistant products. Its excellent photo stability and high absorption coefficient make it a valuable ingredient when a high SPF value is required[2]. BT is an oil-soluble organic compound that is added to sunscreens to absorb UV rays. BT is a broad-spectrum UV absorber, absorbing UVB as well as UVA rays. It has two absorption peaks, 310 and 340 nm. It is highly photo stable. It helps to prevent the photo degradation of other sunscreen actives like avobenzone[3-4]. The chemical structures, molecular formula, molecular mass, IUPAC name and CAS no. of ETZ and BT are shown in Table 1.

Literature survey revealed that several methods are reported such as quantification of ethylhexyl triazone by TLC[5-6], quantification of different UV filters by HPLC[7-8], in vitro test for sunscreens[9-10] and other technique[11] for ETZ and BT either single or in combination with other components. Sun care formulations containing ETZ and BT give very good protection in UVA and UVB range and give higher SPF value. No such sensitive and stability indicating HPLC method is available for quantification of ETZ and BT in sun care formulations. Therefore attempts were made to develop and validate a simple, rapid, sensitive and stability indicating HPLC method for simultaneous estimation of ETZ and BT in sun care formulations. The developed LC method was validated for specificity, method precision, limit of detection and limit of quantification, linearity, accuracy and robustness as per ICH guidelines[12]. This method can be utilized in cosmetic industries to monitor ETZ and BT contents in sun care formulations to assure the quality of the products.

Table 1: Physical properties of ETZ and BT

Molecules details	**ETZ**	**BT**
Structures		
Molecular Formula	$C_{48}H_{66}N_6O_6$	$C_{38}H_{49}N_3O_5$
Molar mass	823.07 g/mol	627.81 g/mol
IUPAC name	4-[[4,6-bis[[4-(2-ethylhexoxy-oxomethyl)phenyl]amino]-1,3,5-triazin-2-yl]amino]benzoic acid 2-ethylhexyl ester	2,2′-[6-(4-methoxyphenyl)- 1,3,5-triazine-2,4-diyl] bis{5-[(2-ethylhexyl)oxy]phenol}
CAS no.	88122-99-0	187393-00-6

MATERIALS AND METHOD

Chemicals and reagents

ETZ (Potency: 99.3%) and BT (Potency: 98%) reference standards, Sun care topical lotions, Tetrahydroufuran (THF) HPLC grade (RFCL, India), 1,4-Dioxane HPLC grade (RFCL, India), Ammonium formate AR grade (SDFCL, India), Formic acid AR grade (Merck, India), Water HPLC grade and 0.45µ Polytetrafluoroethylene (PTFE) membrane filters were provided for the research work by Dr. Reddy's Laboratories, Hyderabad, India.

Equipment and chromatographic conditions

Analysis was performed with Waters Alliance HPLC system connected with PDA detector and Empower 2 software for data acquisition. Separation of ETZ and BT peaks was achieved on Waters symmetry C18, 150 × 3.9 mm, 5 µ as stationary phase and mixture of 10mM ammonium formate (pH 4.50 adjusted with dilute formic acid) and 1,4-dioxane in the ratio of 20:80 (v/v) as mobile phase at flow rate of 1.0 mL/minute with isocratic mode. Simultaneous detection of ETZ and BT was performed at 311 nm. Column temperature was maintained at 40°C. Sample injection volume was 20 µL and total run time of the method was 12 minutes. Mixture of THF and 1,4-dioxane (50:50, v/v) was used as diluent for standard and sample preparation.

Preparation of standard solution

Standard stock solution was prepared by weighing about 75mg each of ETZ and BT reference standard into a 100mL volumetric flask. Added 70 mL of diluent, sonicated to dissolve it and then diluted to volume up to the mark with diluent. 5 mL of stock solution was diluted to 25mL with diluent to prepare working standard solution having a concentration of 150 µg/mL for ETZ and BT. Linearity solutions were prepared by diluting standard stock solution to prepare series of solutions into the concentration range of 0.10-225 µg/mL and 0.12-225 µg/mL for ETZ and BT respectively. Plotted graphs of concentration versus area response for both analytes.

Preparation of sample solution

Weighed sample of sun care product equivalent to 15 mg each of ETZ and BT into a 100 mL of volumetric flask. Then added 70 mL of diluent and sonicated for 20 minutes with intermittent shaking. Cooled the solution at room temperature and then made up to volume with diluent, mixed it. Filtered the solution through 0.45µ PTFE filter and injected into HPLC.

Analytical method validation

The proposed method was validated for specificity (Force degradation study), method precision, limit of detection and limit of quantification, linearity, accuracy and robustness as per ICH guideline[13].

RESULTS AND DISCUSSION

Method development and optimization

In this work, a simple, sensitive and accurate isocratic RP-HPLC method was proposed for simultaneous estimation of ETZ and BT in the presence of degradants peaks and excipients peaks of formulation. In order to obtain better separation with symmetrical peaks of both analytes and degradants peaks, chromatographic conditions i.e. buffer and organic modifier concentrations and stationary phase were optimized. Both analytes are hydrophobic in nature and therefore mobile phase need to have higher organic modifier and to use mid polar stationary phase (HPLC column) for faster elution of both analytes. An isocratic method employed using different compositions of water, acetonitrile and methanol as mobile phase and using different stationary phases (CN, C8 and C18) with different configurations (15 and 25 cm length, 3.9 and 4.6 mm i.d.) for better separation and symmetrical peaks of both analytes. Peaks of ETZ and BT were strongly retained with broader peak shape by using even high concentration of acetonitrile and methanol in mobile phase. And therefore THF and 1,4-dioxane were tried in mobile phase for separation of both analytes. High concentration of THF (>40%) in mobile phase was created baseline noise in chromatogram and extra peaks generation over a period of time. Both analytes were eluted faster with better separation by using mixture of water and 1,4-dioxane as mobile phase and symmetry C18 (150 x 3.9mm, 5µ) as stationary phase but poor peak shape with high tailing were observed for both components. To modify peak symmetry, different buffers

(KH_2PO_4, Acetic acid and Ammonium formate) with different pH were tried in place of water in mobile phase. Better separation with symmetrical peaks of both analytes were observed with 10 mMol./L ammonium formate buffer having pH 4.50 : 1,4-dioxane (20:80, v/v) as mobile phase. ETZ and BT have very good absorbance in the range of 300 – 400nm and therefore 311 nm wavelength was selected for simultaneous quantification of both components based on desired response. Stressed samples were injected into HPLC with developed chromatographic conditions and results enabled that ETZ and BT were found to be well separated from degradants and excipients peaks of formulation, hence method is considered to be a stability indicating. Based on these experiments, final optimized conditions are described below.

Waters symmetry C18, 150 × 3.9 mm, 5 µ was used as stationary phase. Mobile phase consisted of a mixture of 10 mMol./L ammonium formate buffer having pH of 4.50 and 1,4-dioxane (20:80, v/v) was run at 1.0 mL/min on isocratic mode. Column temperature was maintained at 40°C and detection was monitored at 311 nm. Injection volume was 20µL and run time of the method was 15.0 minutes. Typical retention times of ETZ and BT were 7.0 and 11.0 minutes respectively (Figure 1).

In order to achieve desired recovery of ETZ and BT from sun care products, selection of diluent (solvent system) was important to break matrix and extraction of both analytes from the matrix. And therefore different composition of solvents based on solubility of both analytes were tried for recovery study. Desired recovery of ETZ and BT was achieved by using mixture of THF and 1,4-dioxane (50:50, v/v). Based on these experiments, mixture of THF and 1,4-dioxane (50:50, v/v) was finalized as diluent for the proposed method for preparation of standard and sample preparation.

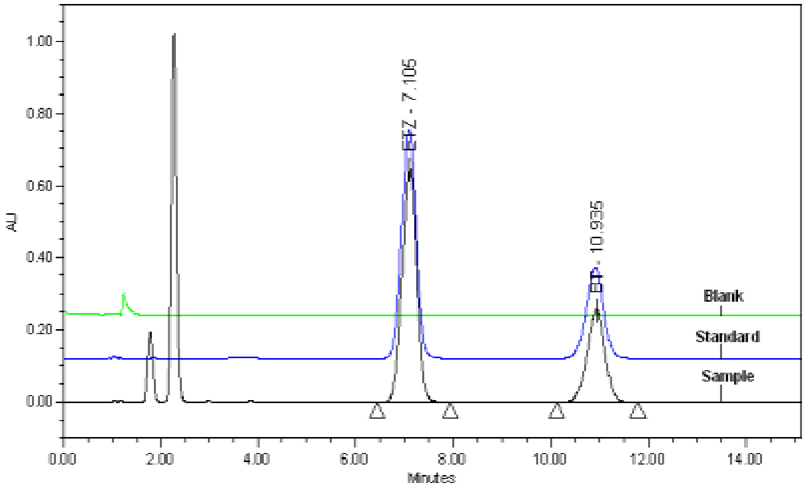


Figure 1: Overlaid chromatogram of Blank, Standard and Sample solution

Analytical Method validation:

Specificity/Force degradation study

Specificity is the ability of the method to measure the analyte response in the presence of its degradants and excipients peaks. In this study, blank was injected into HPLC to check interference at retention time of ETZ and BT peaks and results demonstrate that there was no interference due to blank at retention time of both analytes.

Force degradation studies of sun care lotions were performed to evaluate stability indicating power of the method. Samples were exposed to acid hydrolysis (5 M HCl, 60°C for 3 h), base hydrolysis (5 M NaOH, 60°C for 3 h), oxidation (30% H_2O_2, 60°C for 3 h), thermal degradation (60°C for 12 h) and photolytic degradation (sample exposed to visible light for 1.2 million lux hours and UV light for 200 watt.h/m^2 at 25°C) and analyzed by proposed method. Significant degradation was observed during base hydrolysis (Figure 2), while with other stress conditions no degradation was observed. Peak purity of both analytes were checked in all stress samples. Peak purity of both analytes were passed in all stress samples in the presence of degradants peaks, thus confirms the stability indicating power of the method (Table 2).

Table 2: Results of force degradation study

Degradation conditions	ETZ			BT		
	PA*	PTH#	% Degradation	PA*	PTH#	% Degradation
Control sample	0.040	0.288	-	0.155	0.907	-
Acid hydrolysis sample	0.040	0.278	1.0	0.174	1.062	0.1
Base hydrolysis sample	0.039	0.282	8.3	0.302	1.061	5.7
Oxidative stress sample	0.054	0.286	-	0.139	0.877	-
Thermal stress sample	0.048	0.306	-	0.142	0.908	-
Photolytic stress sample	0.055	0.289	-	0.153	0.883	0.7

PA*: Purity angle, PTH#: Purity threshold, Peak purity criteria: PA<PTH

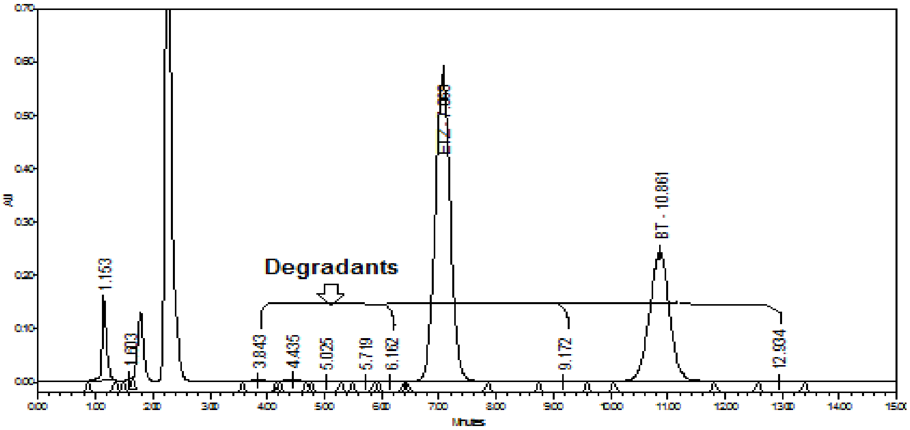


Figure 2: Chromatogram of Base hydrolysis study

Method precision

The precision of the test method was performed in the terms of intra-day and inter-day precision. Intra-day precision was performed by analysis of six sample preparations of sun care lotion as per developed method and calculated % assay of both analytes. Inter-day precision was performed by analysis of six sample preparations of sun care lotion by different analyst using different instrument and on different day as per developed method and calculated % assay of both analytes. Calculated % RSD for the assay of both analytes in replicate samples of intra-day and inter-day precision. % RSD was found to be less than 2.0 for both analytes with respect to intra-day and inter-day precision, thus indicate that method is precise (Table 3).

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

Prepared different series of solutions containing known concentration of ETZ and BT and injected into HPLC as per method. Determined LOD and LOQ as per signal to noise ratio method as per ICH guideline (Table 3).

Linearity:

Linear calibration plots of ETZ and BT were obtained over concentration ranges of 0.10-225 µg/mL and 0.12-225 µg/mL respectively. Plotted graphs of concentration versus area for ETZ and BT (Figure 3). Correlation co-efficient of the linearity plots of both analytes were found >0.999 (Table 3).

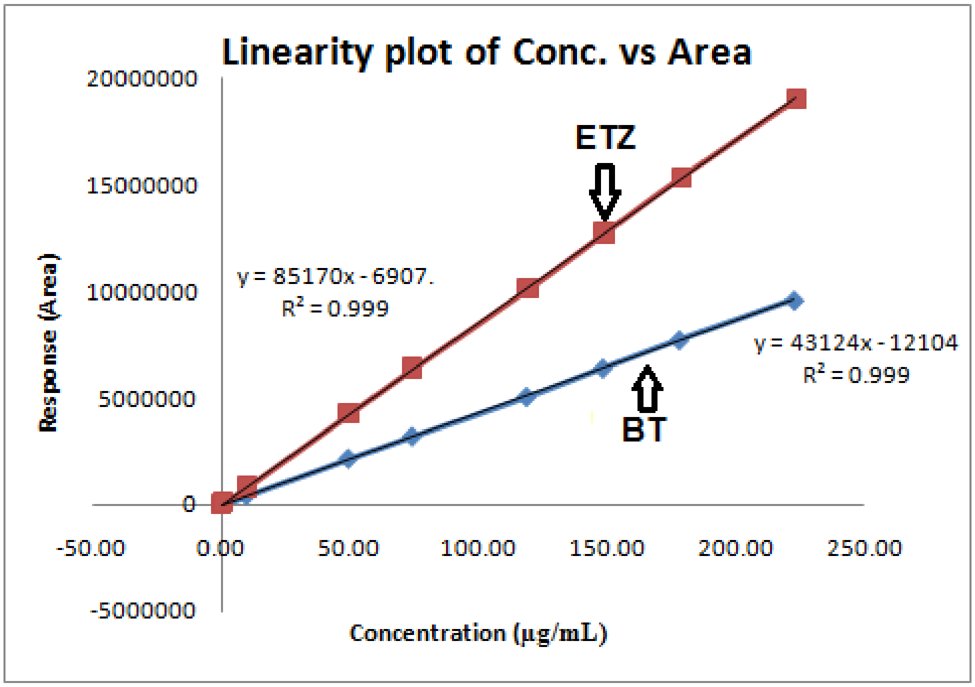


Figure 3: Linearity plot of ETZ and BT

Table 3: **Results of Method precision, LOD/LOQ and Linearity**

Parameters	**BT**	**ETZ**
Method Precision*		
Intra-day Precision (Mean Assay ± SD)	99.4 ± 0.96	100.5 ± 0.96
Inter-day Precision (Mean Assay ± SD)	100 ± 1.47	101.2± 1.48
LOD and LOQ		
LOD	0.04 µg mL^{-1}	0.10 µg mL^{-1}
LOQ	0.12 µg mL^{-1}	0.10 g mL^{-1}
Linearity		
Correlation Co-efficient (r)	0.99996	0.99998
Linearity equation	$y = 43124x – 12104$	$y = 85170x - 6907$
Y intercept Bias in %	0.67	0.67
Concentration range	0.12 - 225 µg mL^{-1}	0.10 - 225 µg mL^{-1}

***n=6 sample preparation**

Accuracy

Accuracy of the proposed method was demonstrated by standard addition method. Samples for accuracy study were prepared by spiking standard stock solution containing ETZ and BT into test sample to achieve 50%, 100% and 150% of the test concentration (ETZ: 150 µg/mL and BT: 150 µg/mL). Prepared samples in triplicates at each level and analyzed as per test method. Calculated % recovery at each level (Table 4).

Table 4: Results of Accuracy

Recovery at % spike level (n=3)	**BT(Mean recovery ± SD)**	**ETZ(Mean recovery ± SD)**
50% level	98.9 ± 1.12	99.6 ± 0.68
100% level	99.2 ± 0.80	100.3 ± 0.46
150% level	99.1 ± 0.55	100.3± 0.76

Robustness

Experiments were designed to demonstrate that how change in column temperature, flow rate of mobile phase and pH of buffer in mobile phase affect the separation of ETZ and BT. Standard solution was injected into HPLC with variation in column temperature of ± 5°C (Actual temperature: 40°C), variation in flow rate of ± 0.1 mL/minute (Actual flow rate: 1.0 mL/minute) and variation in buffer pH ± 0.20 (Actual buffer pH 4.50) from nominal conditions.

Table 5: Results of Robustness study

Robustness conditions$^{\#}$	**USP Tailing factor (NMT 1.50)**		**USP Plate count (NLT 2500)**	
	BT	**ETZ**	**BT**	**ETZ**
Control condition	0.93	1.02	5156	3752
Flow rate 0.9 mL min^{-1}	0.95	1.05	5320	3870
Flow rate 1.1 mL min^{-1}	0.90	0.98	4980	3500
Column Temp. 35°C	0.94	1.03	5145	3612
Column Temp. 45°C	0.95	1.00	4835	3478
4.30 pH Buffer	0.92	1.01	5201	3687
4.70 pH Buffer	0.93	1.03	5356	3711

Replicate standard injections (n=5) injected at each condition

System suitability parameters were checked in each variable conditions (Table 5).

Solution stability of standard and sample preparation

Standard and sample solutions were prepared, kept on bench top and injected into HPLC at initial, after 24 hrs, after 48 hrs and after 96 hrs as per test method. Checked standard correlation for both analytes and assay of both analytes in sample preparation against freshly prepared standard. The standard correlation for both analytes was found between 0.98 to1.02 from initial to 96 hrs. The difference in assay of both analytes in sample preparation from initial to 96 hrs was found within ± 2%. Thus indicate that standard and sample preparation are stable for 96 hrs at room temperature.

CONCLUSION

A simple, sensitive and accurate stability indicating RP-HPLC method was developed and validated for the quantitative analysis of ETZ and BT in sun care formulations. The proposed method was validated and found to be specific, precise, linear, accurate and robust. Accuracy of the method with respect to quantification of both analytes was found > 98.0%. The proposed method was able to quantitate ETZ and BT in the presence of degradants and excipients peaks of formulation, enabling it as stability indicating. This method can be utilized in cosmetic industries to monitor ETZ and BT contents in sun care formulations to assure the quality of the products.

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