Original Article

Development and validation of a reverse phase-liquid chromatographic method for the estimation of butylated hydroxytoluene as antioxidant in paricalcitol hard gelatin capsule formulation dosage form

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Abstract

Introduction: A novel and simple isocratic reverse phase liquid chromatographic (RP-LC) method was developed for the quantitative determination of antioxidant-butylated hydroxy toluene (BHT) in paricalcitol hard gelatin capsule. In the paricalcitol capsule BHT concentration is very low. This method is precisely able to estimate BHT at low concentration at about 0.0039 µg/mL and to separate BHT from paricalcitol main compound and other oil-based excipients. **Materials and Methods:** The method was developed by using ACE-C18 (250 × 4.6 mm) 5-µm column with mobile phase containing a mixture of solvent A (water) and solvent B (methanol) in the ratio of 5:95 v/v, respectively. The flow rate was 0.8 mL/min with column temperature of 45°C and detection wavelength at 277 nm. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness. **Results:** In the precision study the % RSD for the result of BHT was below 1.5% at target concentration level. The limit of detection, limit of quantification are 0.0013 µg/mL and 0.0039 µg/mL, respectively and precision at LOQ level (0.0039 µg/mL) was with 6.2% RSD. The method was linear with concentration rage of 0.0039-0.64 µg/mL with the correlation coefficient greater than 0.999 and % bias at 100% level are within + 2%. The percentage recoveries for BHT were calculated observed from 98.8 to 104.8%. **Conclusion:** The developed method was found to be precise, accurate, linear, selective and robust.

Key words: Antioxidant, butylated hydroxytoluene, paricalcitol capsule, RP—LC, validation

INTRODUCTION

Paricalcitol hard gelatin capsules contain the active ingredient paricalcitol which is a synthetically manufactured analog of calcitriol, the metabolically active form of vitamin D indicated for the prevention and treatment of secondary hyperparathyroidism in chronic kidney disease. Paricalcitol HG capsule oral administration contains 1, 2 or 4 µg of paricalcitol. Use of hard gelatin capsule offers processing convenience in minimizing the hazards of cross-contamination, reduces the need for complex and expensive engineering controls, and assures product uniformity.

Butylated hydroxytoluene (BHT) which is used in paricalcitol HG capsule is a phenolic compound and chemically described as 2, 6-bis (1, 1-dimethylethyl)-4-methylphenol [Figure 1]. Its empirical formula is $C_{15}H_{24}O$, which corresponds to a molecular weight of 220.34. It is available in solid form that is often added as antioxidant in pharmaceutical dosage products.[1,2]

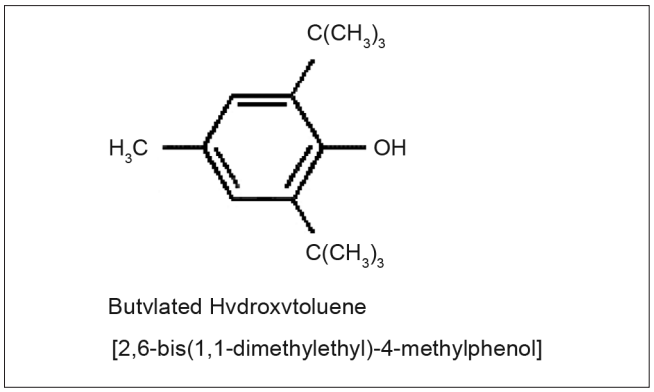

Figure 1: Structure and chemical name of butylated hydroxytoluene

BHT is essentially used to prevent oxidative rancidity in pharmaceutical dosage form.[3] The concentration of BHT depends on the amount of sensitive compounds (α-hydroxy acids, ceramides, lipids, vitamins, oils, and so forth) that are susceptible to oxidation by the oxygen in the atmosphere making it possible for the unstable peroxide radicals.[4,5] BHT is able to inhibit reactions promoted by oxygen, thus avoiding the oxidation and is intended to prevent the appearance of ketones and aldehydes that can give a product a disagreeable smell and rancidity.[5] To prevent formulations from peroxide radicals we must use antioxidant compound which have the ability to neutralize those radicals through the transfer of hydrogen to this radical, stabilizing the antioxidant by resonance.[6,7]

Reversed phase liquid chromatography (RP-LC) with UV/Vis detector is an important analytical technique with strong chromophores that absorb light in the wavelength region from 200 to 800 nm.[8] In literature survey there were several publications and research papers focus on separation methods to detect phenolic antioxidants as BHT by RP-HPLC, GC and by LC-MS.[1,5,9,10-15] Beside the reported method, as per our current knowledge no method is reported by RP-LC at very low concentration of BHT as 0.0039 mcg/mL in paricalcitol capsule. This paper described method is a stability indicating method that can separate BHT from oil-based excipients. The developed LC method was validated with respect to specificity, LOD, LOQ, linearity, precision, accuracy and robustness. Specificity studies were performed on the placebo and drug products to show the stability-indicating nature of the method. These studies were performed in accordance with established ICH guidelines.[16]

MATERIALS AND METHODS

Chemicals and reagents

Samples of paricalcitol HG capsules and BHT were supplied by Dr. Reddy's laboratories limited, Hyderabad, India. The HPLC grade acetonitrile and methanol were from Merck, Mumbai, India. High purity water was prepared by using Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

Equipment

The chromatographic analysis was performed using Waters Alliance 2695 separation module (Waters Corporation, Milford, MA, USA) equipped with 2489 UV/visible detector, degasser, quaternary pump and auto sampler system. The output signal was monitored and processed using Empower 2 software. Ultrasonic sonicator was used for sonication during sample preparation. The pH of the solutions was measured by a pH meter (Mettler-Toledo, Switzerland).

Chromatographic conditions

The method was developed using ACE C18 (250 × 4.6 mm, 5 µm) column with mobile phase containing a mixture of solvent A (water) and Solvent B (Methanol) in the ratio of 50:950 v/v respectively. Isocratic method was used with runtime of 25 minute for sample and 12 minute for standard Solution. The mobile phases were filtered through nylon 0.45-µm membrane filters and degassed in sonicator. The flow rate of the mobile phase was 0.8 mL/min. The column temperature was maintained at 45°C and the eluted compounds were monitored at the wavelength of 277 nm. The injection volume was 500 µl.

Preparation of standard solution

A standard stock solution (Stock A) of BHT were prepared in diluent-1 (water:Acetonitrile, 1:9) with a concentration of 0.2 mg/mL. Working standard solution was prepared from above stock solution (stock A) by further dilution with diluent-2 (water: Acetonitrile, 3:7) to get final concentration of 0.32 µg/mL of BHT.

Preparation of sample solution

Five capsules of paricalcitol was taken and cut the each capsule shell and transferred accurately the whole capsules with content in to a 50-mL volumetric flask. Added 25 mL of diluent-1 (water:Acetonitrile,1:9) and sonicate for about 20 minutes in the sonicator with vigorous intermediate shaking. Allow the flask to stand at room temperature and make the volume up to the mark with diluent-1(water:Acetonitrile, 1:9) and mix well. Further diluted with diluent-2 (water: Acetonitrile, 3:7) to get the concentration of BHT as 0.32 ppm.

RESULTS AND DISCUSSION

Method development and optimization

As BHT concentration is very low, the main objective was to develop a method for detection of BHT and the chromatographic method should able to separate critical closely eluting compounds from BHT, with a shorter run time. The blend containing 0.32 mcg/mL of BHT was used for method optimization. An isocratic method employed using Milli-Q water and methanol in the ratio of 20:80 as mobile phase, Alltima C18 (250 × 4.6 mm) 5-µm column with flow rate of 2.0 mL/min on HPLC equipped with photo diode array detector.60% acetonitrile was used as diluent. BHT peak was merged with excipient peak. To resolve the peak an attempt were made with different ratio of water and methanol in mobile phase by changing Ace C18, 250 × 4.6 mm, 5-µm column. BHT peak was very well resolved but the recovery found in lower side. Therefore to achieve satisfactory recovery, different experiments were conducted in various diluents. Recovery was increased by changing the diluent and sonication time but peak shape was not found symmetrical. Further experiment were conducted by different diluent ratio of water and acetonitrile and decided to keep two diluent, first diluent for extraction of BHT from paricalcitol capsule where organic solvent ratio is more and second diluent to get symmetrical peak shape. Based on these experiments, the conditions were further optimized as described below.

The method was finalized by using ACE C18 (250 × 4.6 mm, 5 µm) column with mobile phase containing a mixture of solvent A (water) and Solvent B (Methanol) in the ratio of 50:950 v/v respectively. The mobile phase was filtered through nylon 0.45-µm membrane filters and degassed in sonicator. Isocratic method was used with runtime of 25 minute for sample and 12minute for standard. Water and acetonitrile in the ratio of 1:9 was used as diluent-1 and water: Acetonitrile in the ratio of 3:7 was used as diluent-2. The flow rate of the mobile phase was 0.8 mL/min. The column temperature was maintained at 45°C and the eluted compound was monitored at the wavelength of 277 nm. The injection volume was 500 µL.

Method validation

The proposed method was validated as per ICH guideline.[17] The following validation characteristics were addressed: system suitability, specificity, precision, limit of detection and quantification, linearity, range, accuracy, solution stability, mobile phase stability and robustness.

System suitability

System suitability was checked for the conformance of suitability and reproducibility of chromatographic system for analysis. The system suitability was evaluated on the basis of USP tailing factor and theoretical plates of BHT and relative standard deviation (RSD) of five injections of standard solution. System suitability was determined before sample analysis from five replicate injections of the standard solution containing 0.32 µg/mL BHT [Figure 2]. The acceptance criteria were % RSD should not be more than 2.0%, USP tailing factor should less than 2.0 and theoretical plate should be more than 3000 for BHT peak from standard solution. All critical parameters tested met the acceptance criteria [Table 1].

Specificity

A specificity study to establish the interference of placebo was conducted. Study was performed on Placebo (Placebo contains without BHT, with Paricalcitol and other excipients) in duplicate equivalent to about the weight of placebo present in portion of test preparation as per test method. Chromatograms of placebo had shown no peaks at the retention time of BHT, this indicates that the excipients used in the formulation do not have any interference in estimation of BHT in Paricalcitol capsules [Figure 3].

Precision

The precision of method was verified by repeatability and intermediate precision at target concentration level (0.32 µg/mL). Repeatability was checked by injecting six individual preparations of BHT in paricalcitol hard gelatin capsule as per test method [Figure 4]. %RSD of result for BHT was calculated. The intermediate precision of the method was also evaluated using different analyst and different instrument and performing the analysis on different days.

The % RSD for the result of BHT in repeatability study was within 1.5% and in intermediate precision study was within 0.2%, which confirms the good precision of

Table 1: System suitability test results

Parameters	Specification	Observed values
Theoretical plates for BHT peak from standard injection	≥3000	13030
The relative standard deviation for peak areas in five standard injections	≤2.0%	0.3
The USP tailing factor for BHT peak	≤2.0	1.1

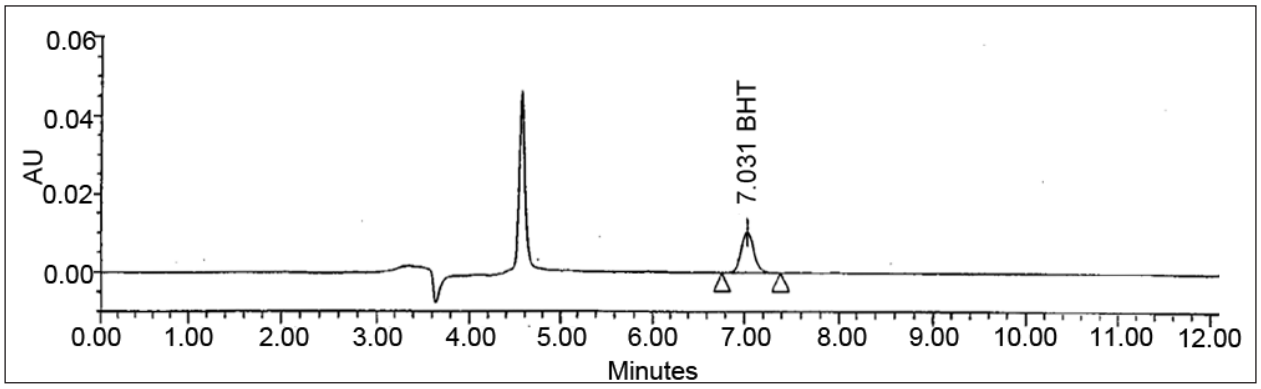

Figure 2: Typical chromatograms of standard solution

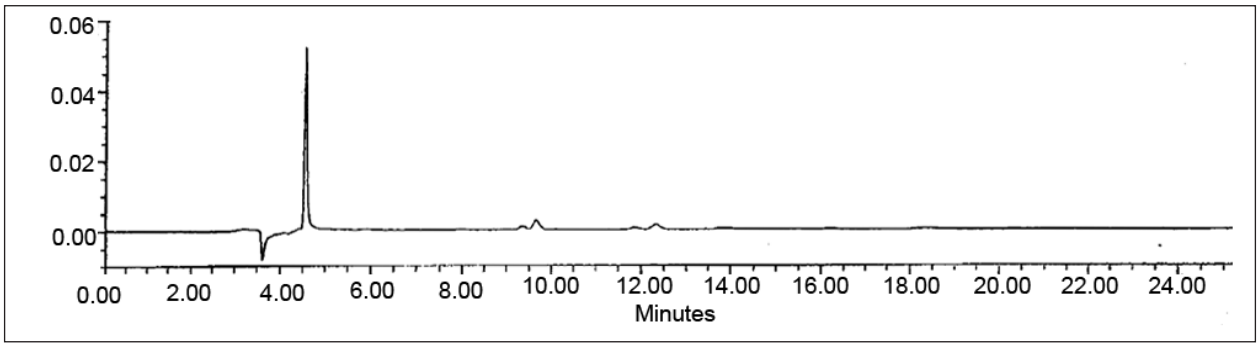

Figure 3: Typical chromatograms of placebo

Table 2: Repeatability and intermediate precision test results

Sample no.	% Butylated hydroxytoluene	
	Repeatability	Intermediate precision
1	96.4	97.6
2	100.7	97.2
3	98.5	97.6
4	99.7	97.9
5	98.0	97.4
6	98.2	97.7
Mean	98.6	97.6
%RSD	1.5	0.2

the method. The %RSD values for BHT are presented in Table 2.

Limits of detection and quantification

The LOD and LOQ for BHT were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of diluted solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six individual preparations of BHT and calculated the %RSD of the area. The determined limit of detection, limit of quantification and precision at LOQ values for BHT are reported in Table 3.

Table 3: Linearity and LOD-LOQ data

Parameter	BHT
LOD (µg/mL)	0.0013
LOQ (µg/mL)	0.0039
Correlation coefficient	0.9999
Intercept (a)	38.3016
Slope (b)	306579.0448
Bias at 100% response	0.04
Precision at LOQ (%RSD)	6.2

Linearity

Linearity test solutions were prepared by diluting the stock solutions to the required concentrations by covering the range from 0.0039 to 0.64 µg/mL of BHT [Table 4]. The solutions were prepared at six concentration levels from LOQ to 200% of specification level (LOQ, 25%. 50%, 100%, 150% and 200%). Calibration curves were plotted between the responses of peak versus analyte concentrations [Figure 5]. The correlation coefficient obtained was greater than 0.999 and % bias at 100% level are within ± 2%. The above result shows that an excellent correlation existed between peak area and concentration of BHT. The coefficient correlation, slope and y-intercept of the calibration curve and bias at 100% response are summarized in Table 3.

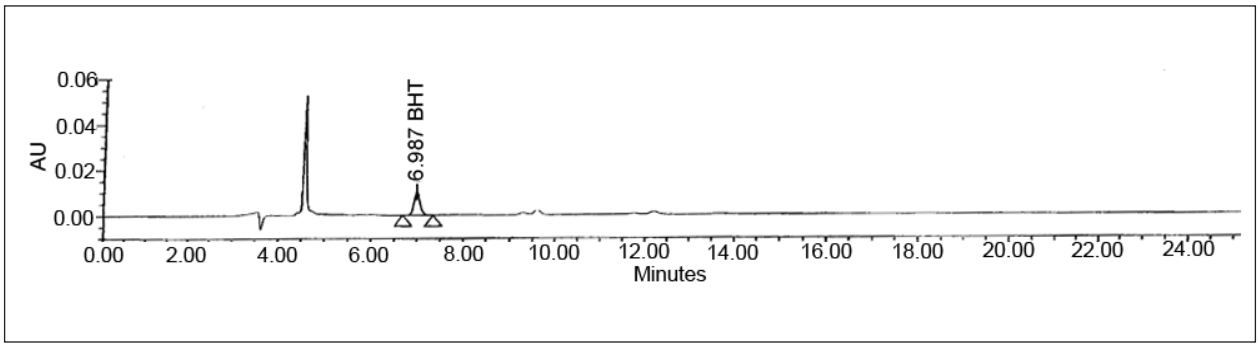

Figure 4: Typical chromatograms of sample solution

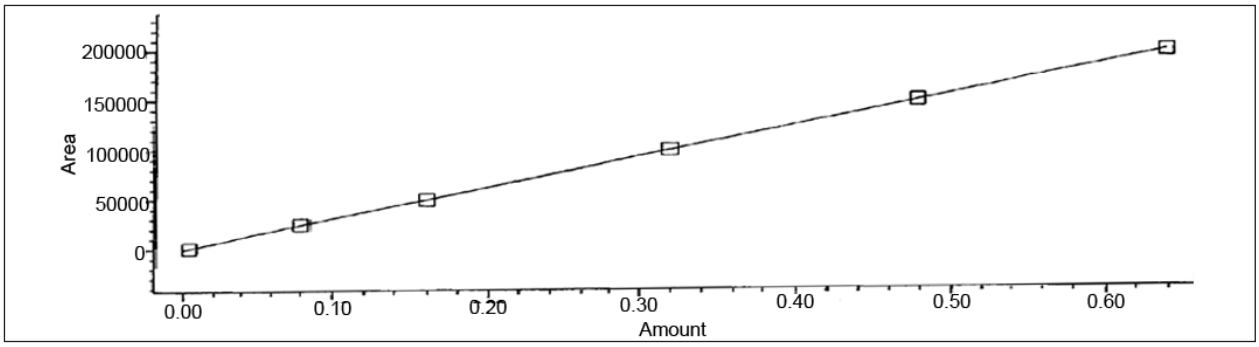

Figure 5: Linearity graph

Table 4: Linearity study concentration of BHT and peak area

Concentration of BHT in µg/mL	Area
0.0039	968
0.0798	24534
0.1596	48964
0.3191	98217
0.4787	147062
0.6382	195323

Accuracy

Accuracy of the method BHT were evaluated in triplicate using six concentration levels LOQ (0.0039), 0.08, 0.16, 0.32, 0.48 and 0.64 µg/mL The percentage recoveries for BHT were calculated and varied from 98.8 to 104.8. The recovery values are presented in Table 5.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and system suitability (SST) parameters for BHT standard were recorded. The variables evaluated in the study were composition of the mobile phase, column temperature and flow rate. The flow rate of the mobile phase was 0.8 mL/min. To study the effect of flow rate on the SST, flow was changed from 0.6 to 1.0 mL/min. The effect of composition of mobile was studied at 90% and 110% of the method organic phase composition. The effect of the column temperature on SST was studied at 40°C and 50°C instead of 45°C. In all the deliberate varied chromatographic conditions, maximum tailing factor for BHT peak from standard solution was 1.1, minimum theoretical plate was 12059 and the %RSD of peak areas was maximum 1.9. The system suitability parameters evaluated are shown in Table 6.

Stability of the solutions and mobile phase

The solution stability of BHT was determined by Keeping test solution and standard solutions in tightly capped volumetric flasks at room temperature for 5 days and measured the amount of BHT at every 24 hrs against freshly prepared standard solution. The stability of mobile phase was also determined by freshly prepared solutions of BHT at 24 hrs interval for 5 days. The mobile was not changed during the study. The variability in the estimation of BHT was within ± 10% during solution stability and mobile phase stability. The results from solution stability and mobile phase stability experiments confirmed that mobile phase was stable up to 5 days and also sample solution and standard solutions were stable up to 5 days on bench top.

Assay result and method application

To check the application of the method three different types of paricalcitol HG capsules formulations were

Table 5: Recovery data

Amount spiked [a]	% Recovery [b]
LOQ	101.6 ± 3.0
50%	102.3 ± 0.7
75%	102.1 ± 1.0
100%	101.7 ± 0.5
125%	102.8 ± 0.3
150%	100.7 ± 0.3

[a]Amount of BHT spiked with respect to specification level; [b]Mean + %RSD for three determinations

Table 6: Robustness results of HPLC method

Variation in chromatographic condition	Observed system suitability parameters		
	Theoretical plate ≥ 3000	USP tailing for BHT peak ≤ 2.0	The %RSD of peak areas [a] ≤ 2.0%
Column temperature 40°C	13652	1.1	0.4
Column temperature 50°C	12329	1.1	1.9
Flow rate 0.6 mL/min	13580	1.1	0.8
Flow rate 1.0 mL/min	12538	1.1	0.7
90% organic composition	12059	1.1	0.7
110% organic composition	13400	1.1	0.4

[a]The relative standard deviation of peak areas in five standard injections

Table 7: Assay results of RP-LC method

Sample	Amount of BHT in mg/cap		%labeled claim ± %RSD
	Labeled	Estimated	
Paricalcitol HG capsule Formulation-1	0.0080	0.0081	101.2 ± 2.3
Paricalcitol HG capsule Formulation-2	0.0160	0.0160	100.0 ± 0.9
Paricalcitol HG capsule Formulation-3	0.0240	0.0242	100.8 ± 1.3

selected where BHT concentration were different. These each sample were analyzed three times as per above-mentioned method and results are found satisfactory which is summarized Table 7.

CONCLUSIONS

A simple and efficient reverse-phase HPLC method was developed and validated for quantitative analysis of Butylated Hydroxy toluene in paricalcitol capsule pharmaceutical dosage forms. The method found to be precise, accurate, linear, robust and rugged during validation. Satisfactory results were obtained from the validation of the method. The method is can be used for routine analysis of production samples and to check the stability of the BHT in paricalcitol capsules.[17]

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