Original Article

Development and Validation of Stability Indicating Method for Estimation of Buparvaquone by Forced Degradation Studies

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ABSTRACT

Aim: A lucid, rapid and precise stability-indicating method was developed by using HPLC for the estimation of Buparvaquone in bulk as well as pharmaceutical dosage form by forced degradation studies. **Materials and Methods:** Princeton C_{18} column (4.6 × 150 mm, 5μ) and mobile phase containing 1% glacial acetic acid and acetonitrile in the proportion of 5:95 v/v was used throughout the study. The flow rate was 0.9 ml/m and the detecting wavelength was kept as 251 nm using the PDA detector. **Results:** The retention time of Buparvaquone was found to be at 8.6 ± 0.5 m. The method developed was validated, as stated in ICH Q2 (R1) guidelines. It was found to be linear within concentration ranging from 2-20 μg/ml having a correlation coefficient 0.999 and other parameters are also under permissible limits. **Conclusion:** Buparvaquone was exposed to different stress conditions like acidic, basic, neutral, thermal, peroxide and also photolytic. Amongst all, the drug was found to be more degraded under basic as well as peroxide conditions.

Key words: Buparvaquone, Forced degradation, Validation, RP-HPLC, Stability.

Key Message: Novel Stability indicating method for the drug buparvaquone has been developed by using HPLC. Forced degradation studies have been carried out and it also reflects the stability of the drug in various stress conditions.

INTRODUCTION

Buparvaquone (BPQ), 2-[(4-tert-butylcyclohexyl)methyl]-3-hydroxynaphthalene-1,4-dione (Figure 1), is a second-generation hydroxynaphthoquinone antiprotozoal drug used for the therapy and prophylaxis of all forms of theileriosis.[1] It is also used in the treatment of Cutaneous and Visceral Leishmaniasis.[2] It was initially developed as an anti-malarial drug[3] and is currently the most promising compound for the treatment of theileriosis.[4]

Buparvaquone had displayed high *in-vitro* activity in the case of *L. donovani* infections with ED50 values in the middle of 0.12 and 0.005 µM. But after subcutaneous injection, the *in-vivo* activity of Buparvaquone was low.[5] Also, the oral bioavailability of BPQ is low due to low aqueous solubility, which is less than 0.03 µg/ml making the drug highly lipophilic. These problems have led the way to the synthesis of water-soluble phosphate prodrugs of BPQ having water solubility higher than 3.5 µg/ml within the pH range of 3.0-7.4.[6-8]

Literature Survey discloses that there are specific techniques available for the estimation of BPQ like RP-HPLC-UV method, Spectrofluorimetric method and Bioanalytical method.[9,10] The current study was designed to develop novel, simple and accurate stability-indicating method[11,12] for the determination of Buparvaquone in bulk and in pharmaceutical preparation.

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MATERIALS AND METHODS

Buparvaquone active pharmaceutical ingredient has been obtained as a gift sample from Dr. Lalatsa Lab, University of Portsmouth, Hampshire, UK. Pharmaceutical formulation in the form of tablets (Buparvaquone 100mg) was purchased from the local market manufactured by Vetbiochem India Pvt Ltd, Pune, Maharashtra, India. All the solvents used were of HPLC grade and reagents were of AR grade.

Solvents and Reagents: Acetonitrile, Glacial acetic acid (GAA), water, NaOH, Concentrated HCl, 30% H_2O_2.

Chromatographic conditions

Shimadzu LC-6AD semi-preparative HPLC was used and the mode of elution was isocratic. The mobile phase consisting of acetonitrile and 1% GAA in the proportion of 95:5 v/v was used with flow rate of 0.9 ml/m and the column used was PRINCETON C_{18} (4.6×150 mm, 5µ). Twenty microliters of the sample have been injected through a loop injector. Detecting wavelength was kept at 251 nm.

Preparation of standard solution

Ten milligram of Buparvaquone was weighed accurately and dissolved in a small quantity of acetonitrile and the final volume was made up to 10ml with the same. It was sonicated for 15 min to get a clear solution (concentration 1000 µg/ml). One ml from the above solution was taken out and diluted up to 100 ml to achieve the final concentration of 10 µg/ml.

Preparation of sample solution

For sample preparation, acetonitrile was used as a solvent for extraction and dilution. Twenty tablets of BPQ were weighed and finely pulverized. An accurately weighed powder of tablet equivalent to 10 mg of Buparvaquone (40.58 mg) was transferred into a 10ml volumetric flask. About 5 ml of acetonitrile was added and the mixture was sonicated for 15 min. The solution was allowed to cool at room temperature and then diluted with acetonitrile until it reaches the mark (stock solution 1000 µg/ml). The above solution is then filtered through Whatman filter paper having grade I. One milliliter of the filtrate was moved to a 100 ml volumetric flask and the volume was made up to the mark using acetonitrile to obtain a concentration of 10 µg/ml.

Method development

For the estimation of Buparvaquone, the pure drug was injected in the HPLC system and run by using the mobile phase. In order to select a mobile phase initially, various organic solvents in a single and combinations were tried in order to obtain a sharp peak of Buparvaquone with adequate retention. The mobile phase containing plane methanol, plane acetonitrile, a combination of methanol: formic acid, acetonitrile: water, methanol: acetonitrile, formic acid: acetonitrile Combination of phosphate buffer: methanol: acetonitrile at pH range 7 ±05 was tried, but satisfactory results were not obtained. Ammonium acetate buffer: methanol and Ammonium acetate buffer: acetonitrile with variable pH range 3.8, 4, 5, 5.8 and different compositions of Ammonium acetate buffer: acetonitrile 10:90, 80:20, 30:70, 40:60, 50:50 was tried. For satisfactory retention time, the flow rate was adjusted at different rates. The mobile phase containing acetonitrile and 1% Glacial acetic acid in the ratio of 95:5 v/v with a flow rate of 0.9 ml/m was found to be competent as it gives a sharp, symmetrical peak with adequate retention.

Validation of method

Validation of the method was carried out as stated in ICH guidelines. The parameters which were evaluated are Linearity, Precision, system suitability, Robustness, Accuracy, Limit of detection (LOD) and Limit of quantitation (LOQ).

System Suitability parameters

The chromatographic conditions were set according to the optimized parameters and the mobile phase was allowed to pass through a stationary phase to get a steady baseline. Six replicate injections of working standard solutions were injected independently and the values were noted.

Linearity

Linearity was studied by injecting the different concentrations of drugs ranging from 2-20 µg/ml. The graph of concentration versus area has been plotted and the correlation coefficient (r^2) is recorded.

Accuracy

Accuracy of the method was confirmed on the basis of recovery studies carried out by using the standard addition method at three different levels of the labeled claim (80, 100 and 120% w/w). Three replicate injection of each level was made separately and values were recorded.

Precision

The precision of the method has been done by injecting the solution on the same day (Intraday), different day (Interday) and also by using another analyst.

Robustness

The robustness of the proposed method was studied by making small alterations in wavelength and also in the acetonitrile content of the mobile phase. Values were recorded as %RSD.

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were computed using the formula

LOD= 3.3 × (σ)/s

LOQ= 10 × (σ)/s

Where,

s means Slope

σ means Standard Deviation (SD)

Forced Degradation Studies

Preparation of Forced Degradation Sample

For the preparation of forced degradation samples, Buparvaquone was subjected to different stress conditions like acidic, alkaline, oxidative, thermal, neutral and photolytic. The stress conditions for each of the degradation types were optimized on trial and error basis to obtain about 5 to 20% degradation.

Acid hydrolysis

Ten mg of Buparvaquone was weighed accurately and solubilized in 10 ml acetonitrile to obtain a concentration of 1000 µg/ml. Then from the above stock solution, 1 ml was diluted up to 10 ml with 2N HCl. This solution is heated under reflux for 18 hr. One ml from the above-heated solution was neutralized with 2N NaOH and again diluted with acetonitrile to achieve a concentration of 10µg/ml.

Alkaline hydrolysis

Ten mg of BPQ was weighed accurately and then dissolved in 10 ml acetonitrile in order to obtain a concentration of 1000 µg/ml. Then 1 ml from the above-prepared solution was diluted up to 10 ml with 1N NaOH. This solution is heated under reflux for 8 hr.

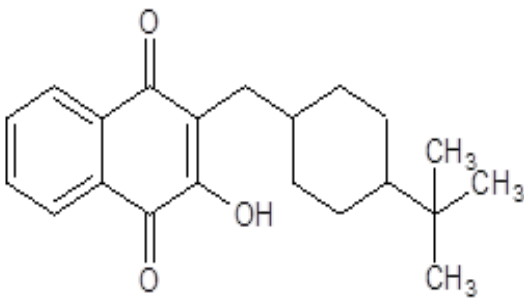

Figure 1: Structure of Buparvaquone.

One ml from the above-heated solution was neutralized with 1N HCl and then again diluted with acetonitrile to achieve the final concentration, which is 10µg/ml.

Oxidative degradation

Ten mg of BPQ was weighed accurately and then solubilized using 10 ml acetonitrile in order to obtain a concentration of 1000 µg/ml. Then 1 ml from the above solution was diluted up to 10 ml with 30% H_2O_2. The mixture was placed at room temperature for 24 hr. And to achieve the concentration of 10 µg/ml, one ml from the resultant solution was diluted using acetonitrile.

Neutral hydrolysis

Ten mg of BPQ was weighed accurately and solubilized in 10 ml acetonitrile to obtain a concentration of 1000 µg/ml. Then 1 ml from the above-prepared solution was diluted up to 10 ml with water. The mixture was refluxed on a water bath at 80°C for 24 hr. One ml from the resultant solution was diluted by using acetonitrile to achieve a concentration of 10 µg/ml.

Thermal degradation

An accurately weighed 10 mg of Buparvaquone was kept in an oven at 80°C for 4 hr. After 4 hr, the resultant sample was solubilized in 10 ml acetonitrile to obtain a concentration of 1000 µg/ml. One ml from it was again diluted up to 10 ml with acetonitrile in order to gain the concentration of 100 µg/ml. Then again, 1 ml was taken and diluted up to 10 ml in order to achieve the required concentration of 10 µg/ml.

Photolytic degradation

Ten mg of BPQ was weighed accurately and kept in sunlight for 15 days. After 15 days, the resultant sample was diluted by using 10 ml acetonitrile to obtain a concentration of 1000 µg/ml. One ml from it was then again diluted using acetonitrile up to 10 ml to achieve 100 µg/ml concentration. Then again, 1 ml was taken and diluted up to 10 ml in order to gain the required concentration of 10 µg/ml.

RESULTS AND DISCUSSION

Method development

Buparvaquone can be easily analyzed by RP-HPLC using the PRINCETON C_{18} column (4.6×150 mm, 5µ) along with the mobile phase, which is acetonitrile and 1% GAA (95:5 v/v) at a wavelength of 251 nm. The retention time (t_R) of the drug was found at 8.62 min. The flow rate was kept as 0.9 ml/m and the total time of analysis was less than 20 m. The chromatogram obtained for Buparvaquone is shown in Figure 2.

Method ValidationSystem Suitability

From the six replicates of samples injected, it was found that %RSD was under permissible limits, which is less than 2%. The results are listed in Table 1.

Linearity

From the calibration curve (shown in Figure 3), it was found that the method is linear within the concentration ranging from 2-20 µg/ml having a correlation coefficient (R^2) of 0.999. The results are summarized in Table 2.

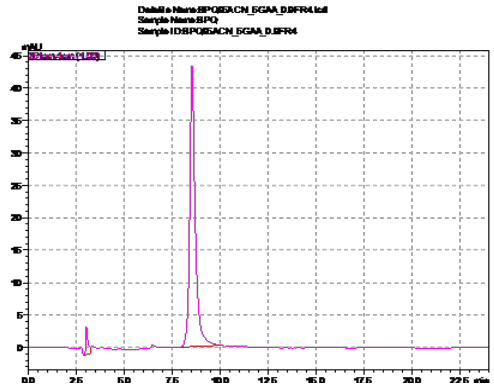

Figure 2: HPLC Chromatogram of Buparvaquone.

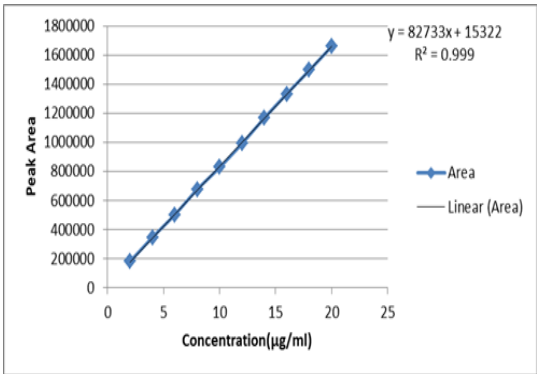

Figure 3: Calibration curve of Buparvaquone.

Table 1: Results of System suitability parameters.

Sr. No.	Retention Time (m)	No. of Theoretical Plates	Capacity Factor	Peak Area
1.	8.63	5368	1.351	859494
2.	8.61	5415	1.281	858245
3.	8.64	5394	1.378	858697
4.	8.63	5376	1.328	859128
5.	8.62	5408	1.295	859492
6.	8.63	5365	1.346	858594
Mean	8.62	5387.66	1.329	858941.7
± SD	0.010328	21.15341	0.036417	511.6126
% RSD	0.119721	0.392627	1.738431	0.0595

Accuracy

Recovery studies have been done with the standard addition method and %Recovery near 100% indicates the accuracy of the proposed method. Table 3 shows the results of accuracy.

Precision

Precision studies have been done by estimating the drug concentration and calculating the %RSD on Interday, Intraday and by using different analysts. Interday precision was found to be 0.05%; intraday precision was 0.02% and by using different analysts, the %RSD was found to be 0.09%. The results are shown in Table 4.

Robustness

The proposed method was found to be robust by changing its scanning wavelength and also changing the acetonitrile content in the mobile phase, as shown in Table 5. Results for robustness were shown in Table 6.

LOD and LOQ

LOD and LOQ determination were done with the method based on standard deviation and calibration curve slope. The results were listed in Table 7.

Assay

%Assay was calculated (Table 8) by injecting the sample solution of 10µg/ml in the HPLC system and the area was noted. From that area of sample and area of standard Buparvaquone has been estimated by using the following formula and the summary of validation parameters were shown in Table 9.

$$\% \text{ Assay} = \frac{\text{Asam} \times \text{Cstd} \times \text{DF} \times \text{Avg.Wt.}}{\text{Astd} \times \text{Wt.taken} \times \text{LC}}$$

Where,

A_{sam} = Sample area
A_{std} = Area of standard
C_{std} = Concentration of standard, µg/ml
DF = Dilution Factor
Avg. Wt = Average Weight of tablets
Wt. taken = Weight of tablet powder taken
LC = Labeled Claim

Table 2: Linearity parameters for Buparvaquone.

Parameters	Buparvaquone
Linear dynamic range (µg/ml)	2-20
Equation	Y= 82733X + 15322
Slope	82733
Intercept	15322
Correlation coefficient (R^2)	0.999

Table 3: Results of Accuracy studies.

Buparvaquone tablets (Avg. Wt. 405.83 mg for 100 mg of Buparvaquone)						
Level	Wt. of sample taken (mg)	Amount of standard added	Area*		Total drug estimated (mg)	% Recovery
			Sample	Standard		
80%	40.61	8.0	1547089	859494	17.92	99.06
100%	40.59	10.0	1718988	859494	19.96	99.64
120%	40.60	12.0	1890886	859494	21.99	99.99
*Mean of 3 readings				Overall	Mean	99.56
					±SD	0.4691
					%RSD	0.4712

Table 4: Results of Precision studies.

Sr. No.	Obs.	% Drug estimation		
		Interday	Intraday	Different Analysts
1.	I	99.96	99.95	99.51
2.	II	99.85	99.99	99.46
3.	III	99.90	99.98	99.62
4.	IV	99.87	99.96	99.43
5.	V	99.92	99.98	99.55
6.	VI	99.84	99.97	99.58
	Mean	99.90	99.98	99.53
	±S.D.	0.0542	0.0214	0.0953
	%R.S.D.	0.0543	0.0214	0.0970

Table 5: Parameters for Robustness study.

Parameter	- Level	Nominal	+ Level
Change in Scanning Wavelength	249 nm	251 nm	253 nm
Change in Acetonitrile content in total mobile phase	93.00 ml	95.00 ml	97.00 ml

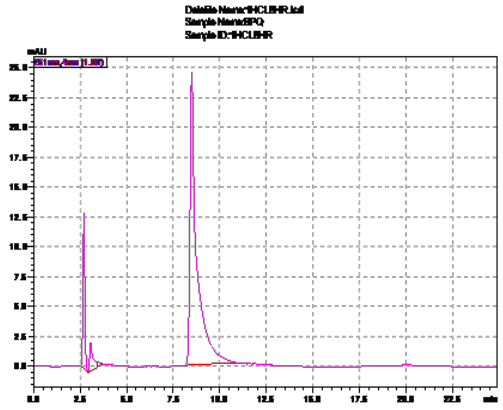

Figure 4: (a) Chromatogram of Buparvaquone after acid hydrolysis.

Table 6: Results of Robustness parameters by HPLC.

Buparvaquone Tablets (Avg. Wt. 405.83 mg for 100mg of Buparvaquone)				
	Wavelength (251±2nm)		Acetonitrile content in mobile phase [ACN : 1% GAA (95 : 5 v/v)]	
	249nm	253nm	93ml ACN	97ml ACN
Mean	100.51	99.89	99.86	100.15
±SD	0.2595	0.6644	0.412	0.2605
%RSD	0.2501	0.6456	0.413	0.2594

Table 7: Results of LOD and LOQ studies.

LOD (µg/ml)	0.47
LOQ(µg/ml)	1.43

Table 8: Assay of marketed formulation.

Buparvaquone tablets (Avg. Wt. 405.83 mg for 100 mg of Buparvaquone)				
Sr. No.	Sample weight (mg)	Area		% Assay
		Standard	Sample	
1	40.58	859494	859218	99.97
2	40.54	858245	858341	100.11
3	40.55	858697	858498	100.05
4	40.58	859128	858998	99.99
5	40.55	859492	859259	100.05
			Mean	100.03
			SD±	0.0569
			%RSD	0.0569

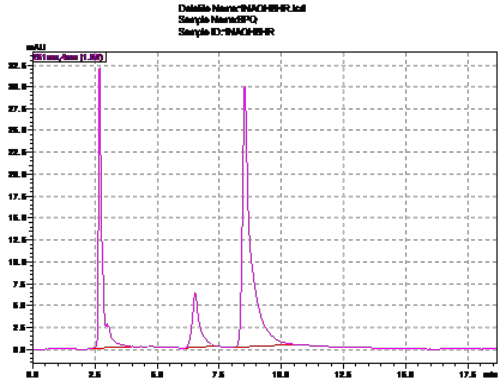

Figure 4: (b) Chromatogram of Buparvaquone after alkaline hydrolysis.

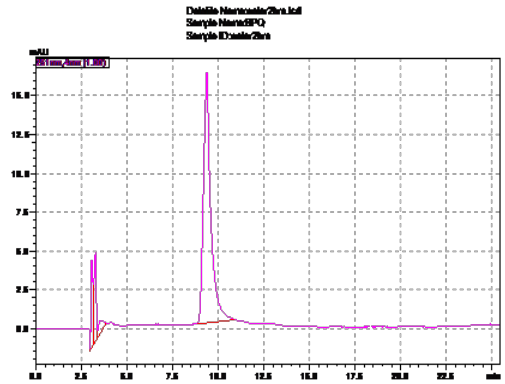

Figure 4: (d) Chromatogram of Buparvaquone after oxidative degradation.

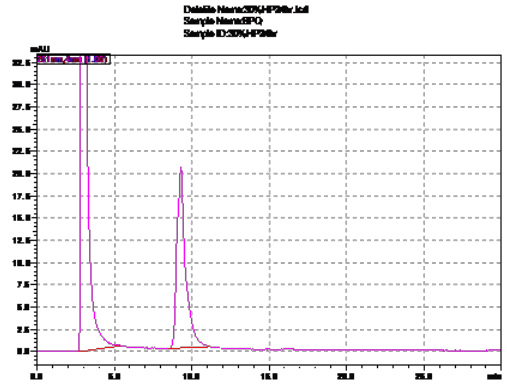

Figure 4: (C) Chromatogram of Buparvaquone after oxidative degradation.

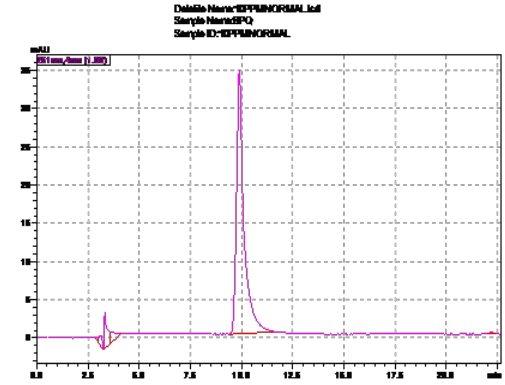

Figure 4: (e) Chromatogram of Buparvaquone after thermal degradation.

Table 9: Summary of Validation Parameters.

Parameters	Observation
Linearity and Range	2-20 µg/ml
Regression equation	Y= 82733X + 15322
Intercept	15322
Slope	82733
Correlation coefficient (r^2)	0.999
% Recovery	99.56%
Precision Intraday (%RSD)	0.02%
Precision Interday (%RSD)	0.05%
Precision Different analyst (%RSD)	0.09%
Robustness	Robust
LOD	0.47 µg/ml
LOQ	1.43 µg/ml
% Assay	100.03%

Forced degradation studies

Forced degradation studies were carried out in order to identify the degradant products. Forced degradation of Buparvaquone was executed under stress conditions of acid, base, oxidation, neutral, thermal and photolytic

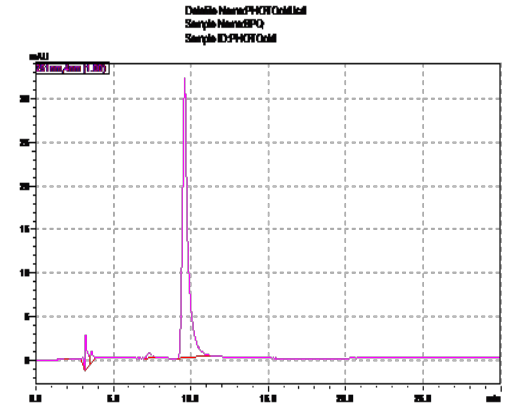

Figure 4: (f) Chromatogram of Buparvaquone after Photolytic degradation.

degradation and the chromatograms are depicted in Figure 4 (a-f). From the results (Table 10), it was clear that the drug was found to be more degraded under alkaline and oxidative conditions.

CONCLUSION

The proposed stability-indicating method was found to be simple, accurate, linear and precise for the estimation of Buparvaquone and the method was

Table 10: Results of forced degradation studies of Buparvaquone.

	A	B	N	O	T	P
Concentration of stressor	2N HCl	1N NaOH	H_2O	30% H_2O_2	--	Sunlight
Duration of stress conditions	Reflux, 18 h	Reflux, 8 h	Reflux, 24 h	RT, 24 h	80ºC, 4 h	15 days
t_R of Degraded Products(m)	8.6	6.5, 8.6	9.2	9.4	9.8	9.2
% of active Buparvaquone	95.05	80.55	92.84	66.85	98.15	92.06

Key; A: acid; N: neutral; B: base; O: oxidative; T: thermal; P: photolytic

validated according to the guidelines of ICH. The drug gets more degraded under alkaline and oxidative conditions. This method can be utilized for the routine analysis of Buparvaquone in bulk and in pharmaceutical preparation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

HCl: Hydrochloric acid; **NaOH:** Sodium hydroxide; **H_2O_2:** Hydrogen Peroxide; **GAA:** Glacial acetic acid; **RSD:** Relative standard deviation.

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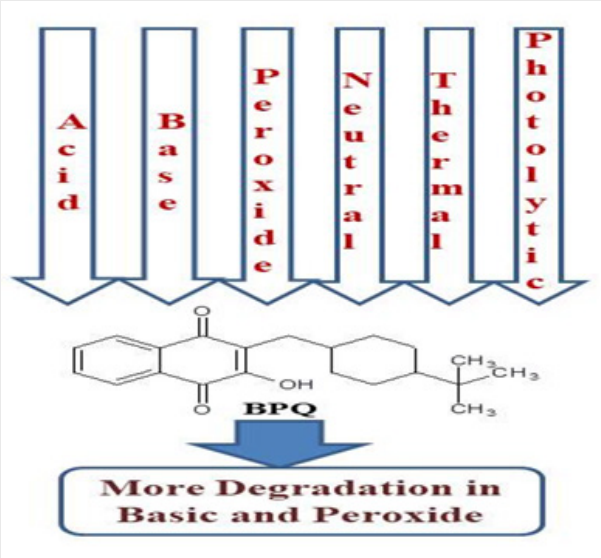
PICTORIAL ABSTRACT



SUMMARY

The drug buparvaquone gets degraded when it is subjected to various stress conditions. The intensity of degradation is more in basic and oxidative conditions.

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