

Development of Stability Indicating TLC-Densitometry Method of Edaravone Using QbD Approach: Degradation Kinetic Study

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ABSTRACT

Objective: The objective of present method was to utilize risk based and systematic Quality by design approach for development of selective, sensitive, precise, accurate and robust stability-indicating TLC-densitometry for quantification of Edaravone and its degradation products. **Method:** The TLC-densitometric analysis was carried out in the absorbance mode at 244 nm using solvent system petroleum ether: ethyl acetate: glacial acetic acid (6ml:4ml:10 μ l v/v/v). This system was found to give compact and well resolved spot for Edaravone at an R_f value of 0.46 $\pm$ 0.21. **Results:** Edaravone undergoes significant degradation when subjected to stress degradation in acid, base, neutral, oxidative, photolytic, dry heat induced and accelerated humidity/temperature degradation conditions. The method was validated according to ICH guideline. Linearity was found in the range of 04-24 μ g/band. The LOD and LOQ for Edaravone were 0.327 μ g/band and 0.989 μ g/band respectively. No interference was observed from excipients in formulation as well as degradation product, indicating specificity of the method. Moreover, the proposed method was also utilized to investigate the kinetics of acid, base, neutral and oxidative degradation process at different concentrations and temperatures. The kinetics of degradation profile was determined using linear and nonlinear regression analysis. The rate constants and half-life were calculated.

Key words: Edaravone, CNX approach, QbD, SIAMs, TLC-densitometry.

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INTRODUCTION

Edaravone (EDA, Radicut®, MCI-186, developed by Mitsubishi Tanabe Pharma Corporation, Osaka, Japan) has wide therapeutic time window.¹ EDA (3-methyl-1-phenyl-2-pyrazolin-5-one) is neuroprotective agent that has been widely used for the treatment of acute embolic stroke in Japan since 2001.² This compound possesses potent free radical scavenging and antioxidant actions by inhibiting hydroxyl radical dependent and independent lipid peroxidation.^{3,4} Unlike other free radical scavengers, EDA (Figure 1) readily crosses the blood-brain barrier.⁵ Previously, it was reported that zEDA may be potentially useful in prevention of many diseases occurred by ROS.^{6,7}

Some methods have been reported for estimation of EDA. These include UV spectrophotometric,⁸ fluorescent assay,⁹ HPLC,¹⁰⁻¹² HPTLC¹³ and LC-MS/MS.¹⁴ The HPTLC method was developed for *in vitro* Estimation of EDA in human plasma¹³ and to the best of our knowledge no Stability indicating assay method have ever been reported. Hence the aim of present study was to develop stability indicating TLC-densitometry method for quantification of EDA and its degradation products (DPs) by implementing systematic QbD approach and to investigate the kinetics of degradation.



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EXPERIMENTAL

Chemicals and reagents

The EDA bulk drug and its formulation (ARAVON IV infusion by Sun Pharmaceuticals Ind. Ltd. containing 1.5 mg/ml of EDA) were purchased from Sigma Aldrich Co, St Louis, USA and local pharmacy respectively. AR grade petroleum ether, ethyl acetate, glacial acetic acid and methanol were procured from Spectrochem Pvt. Ltd., Mumbai. Precoated silica gel aluminum plate 60F₂₅₄ (20 X 20 cm) were procured from Merck, Darmstadt, Germany.

Instrumentation and chromatographic conditions

TLC-densitometry was carried out with a CAMAG TLC Scanner 3 fitted with win CATS 1.4.0 chromatography manager software and data analysis was performed with Design Expert 7.0 software. Chromatographic separation was achieved on pre-coated silica gel aluminum plate 60F₂₅₄ (20X20 cm; Merck, Darmstadt, Germany), that were prewashed by methanol and activated at 80°C for 20 m prior to chromatographic separation. The samples were pre-filtered through a 0.2 µ nylon membrane syringe filter before application. The application rate was maintained constantly at 150 nL s⁻¹ and the bands spaces were maintained automatically by the software. Samples were applied to the pre-coated TLC plates using spray on technique of CAMAG LINOMAT V under the flow of nitrogen gas and developed in a CAMAG 20 cm X 20 cm twin trough glass chambers pre-saturated with the mobile phase to a distance of 89 mm. The mobile phase consisted of petroleum ether-ethyl acetate-glacial acetic acid (GAA) (6 ml: 4 ml: 10 µl v/v/v). After development the TLC plates were dried and scanned on Camag TLC scanner 3 in the absorbance mode at 244 nm.

Preparation of standard, stock and sample solutions

Stock solutions were prepared in acetonitrile (1 mg/ml) for quantitation of the EDA in bulk and in commercial dosage forms (Aravon injection, Sun Pharmaceuticals Ind. Ltd.). To analyze the stressed samples suitable dilutions were made in mobile phase to obtain the final concentration of 12 µg/band with respect to EDA. Same aliquots of EDA were prepared for recovery studies and assay of marketed formulation.

Preparation of degradation products (DPs)

For the stress degradation studies, different stress conditions were used for EDA bulk drug. Placebo samples (without drug) were also prepared for comparison with the stress degradation samples. 1 mg/ml of EDA in

freshly prepared 0.05 N HCl/ 0.8 N NaOH was prepared and was refluxed at 80°C in dark for 180 m for acid and base degradation. For neutral degradation sample was prepared in double distilled water and refluxed at 80°C in dark for 7 hrs. Aliquot of 2 mL of these samples were withdrawn neutralized with NaOH/HCl and stored in freezer before analysis. 1 mg/ml of EDA was prepared in 6% H₂O₂ by the aid of ultra-sonication and was kept at room temperature in dark for 45 m for oxidative degradation. For photolytic degradation solution of 1 mg/ml of EDA in acetonitrile was exposed to 5382 LUX and 144UW/cm² for 21 days. Solid drug was spread in 1mm thickness on a petridish and placed in oven at 80°C for 21 days under dry heat condition in the dark for dry heat induced degradation. For thermal-Humidity induced degradation solid drug was placed in Stability Chamber at 40°C±2° C and 75±5 % RH for 21 days.

All the degradation samples were suitably diluted with mobile phase to make final concentration of 12 µg/band, filtered using 0.2 µ nylon membrane syringe filter before application.

Method development and optimization by risk based and QbD approach

Analytical Target Profile (ATP)

The Analytical Target Profile (ATP) of the present work was to develop stability indicating TLC-densitometry method that shows well resolved chromatogram of EDA and DPs.

Preliminary investigations

To get separation among EDA and DPs various non polar solvents were tried along with ethyl acetate because when ethyl acetate was used alone, all components were travelled with mobile phase. Chloroform, hexane, petroleum ether and toluene were tried among nonpolar organic phase. Petroleum ether gives satisfactory result. To remove tailing and quenching of spot glacial acetic acid was added as third component.

Risk assessment by cause- effect relationship and CNX approach

The knowledge accumulated during the preliminary investigation provides inputs for risk assessment by using tools like Ishikawa/Fishbone diagram and Cause-Effect Risk Assessment Matrix with CNX¹⁵ approach.

Design of Experiments

Box-Behnken design (BBD) design matrix with 17 runs including five center points was used to evaluate the main and interaction effects of the significant factors selected after CNX risk assessment.

Method validation

The present method was validated according to the ICH Q2(R1) guideline.¹⁶

Application of developed HPTLC method

The marketed formulation was analyzed for drug content to determine the possibility of excipient interference. Stress degradation was carried out in the same way as described for bulk drug and % degradation was calculated.

Degradation kinetics study

EDA solutions were prepared at a concentration of 1mg/mL with variable strength of acid (HCl), Base (NaOH) and H₂O₂ for Hydrolysis and oxidative degradation and were stressed at variable temperature for variable time period for kinetic study. Aliquots of 0.5 ml of the sample solutions were withdrawn at different time intervals, neutralized with equivalent strength of NaOH/HCl. Suitable dilutions were made to produce concentration C₀=12 µg/spot, filtered through 0.2 µ membrane syringe filter, applied, developed and scanned by optimized chromatographic conditions. The concentrations of drug remaining were calculated from the formula: % Deg= [initial area of untreated stock solution – reduced area of treated stock solution]/ Initial area of untreated stock solution * 100

The degradation rate kinetics were determined using linear and nonlinear regression analysis. The rate constant (K_{obs}), half-life (t_{1/2}) and activation energy (E_a) were calculated. In the present study linear and nonlinear fit function from GraphPad Prism program was used.

RESULT AND DISCUSSION

Method development and optimization by Risk based and QbD approach

The ishikawa diagram (Figure 2) shows the variables that may affect the method performance characteristics. In the present study, a Cause-Effect Risk Assessment Matrix with CNX approach was utilized (supplementary file 1). Based on risk assessment three CMVs were identified to have significant influence on method performance. These are Mobile phase composition (MPC), chamber saturation time (CST) and migration distances (MD), that were further subjected to BBD to identify optimized chromatographic condition. Table 1 shows the factors with their levels and selected responses used for BBD. The matrixes of BBD with their measured responses are provided in supplementary file 2.

Statistical Analysis and Inferences

The ANOVA (Table 2) results showed highly statistical significant difference between the model terms

(p<0.05), high r² value, insignificant lack of fit, and lower values of PRESS. The significant factors were selected from half normal probability plot. Further, the polynomial regression equations generated for each of the studied responses RS1, RS2 and Rf were assessed for the model terms and interacting variables. Coefficients for each model terms and each factor were analyzed to identify influence of each variable and their interactions on magnitude of responses (supplementary

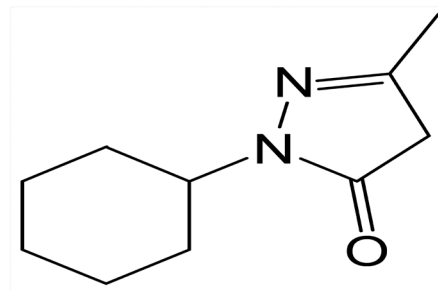


Figure 1: Structure of Edaravone

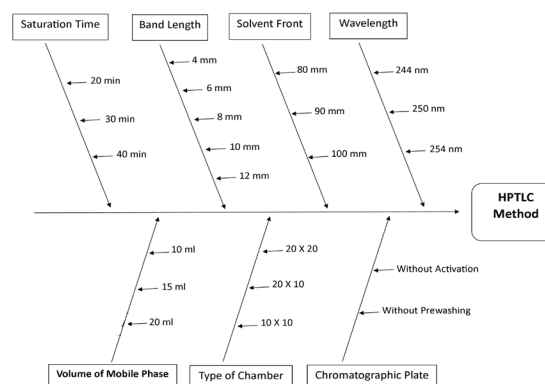


Figure 2: Ishikawa or fish bone diagram of HPTLC method.

Table 1: Variables and their levels for Box Behenken Design

Factors	Coded Levels	Actual Levels
A: Chamber Saturation time (min)	-1	20
	1	40
B: Migration distance (mm)	-1	80
	1	100
C: Mobile phase composition (Ratio of Petroleum Ether)	-1	4
	1	8
Responses		Constraints
R1: resolution between DP-1 and DP-2 (RS1)		0.08≤ R1≥0.1
R2: : resolution between DP-2 and DP-3 (RS2)		0.08≤ R2≥0.1
R3: Retardation Factor of EDA (Rf)		0.3≤ R3≥0.5

file 3). Model equations for the studied response variables are as given below:

$$RS1 = +0.12 - 5.625E-003 * A + 0.054 * C - 9.500E-003 * A * C - 0.019 * A^2 - 0.051 * C^2$$

$$RS2 = +0.10 + 0.015 * A + 7.125E-003 * B - 0.042 * C + 9.500E-003 * A * C + 0.015 * B * C - 8.000E-003 * B^2 - 0.027 * C^2$$

$$Rf = +0.44 + 0.087 * B - 0.28 * C - 0.049 * B^2 + 0.19 * C^2$$

The magnitude of the coefficients in the equation and the p-value (<0.001) indicated that the factors A and C significantly affected the response RS1. The significant interaction effect was AC. For response RS2 all factors i. e. A, B, C affected significantly and significant interaction effects were AC and BC. For response Rf, B and C significantly affected, and interaction effects were not observed. There is reasonable good agreement between the adjusted and predicted r^2 value for all responses. The optimum conditions were calculated by using numerical optimization. To achieve the composite desirability (di)

the response criteria were set as, in range (0.09-0.1) for RS1 and RS2 and in range (0.3-0.5), for Rf. Derringer's desirability was calculated and the final optimum solution was selected (i.e. CST-22 m, MD- 89 mm, MPC-5.8). The desirability and overlay plots are provided in supplementary file 4. The experimental results along with 95% Confidence interval (CI) and prediction interval (PI) values for selected responses were lie within range. The fairly good agreement between experimental and predicted results shows the robustness of the selected model.

The reliability of the selected model was evaluated using cross-validation. The predicted, experimental values and % bias for responses are provided in supplementary file 5. Lower values of % bias indicate the validity of model and were calculated by the Equation: Bias = (Predicted value – Experimental value)/ Predicted value

The optimized conditions obtained by using QbD were then used to obtain the final chromatograms that

Table 2: ANOVA results showing the effect of independent variables on the responses

Response	Model	SS	DF	MS	F-value	p-value	PRESS	r2	Adj- r2	Pred- r2	AP
RS1	RQM	0.037	5	7.441E-003	48.61	< 0.0001	6.689E-003	0.9567	0.9370	0.8280	17.860
RS2	RQM	0.021	7	3.026E-003	70.59	< 0.0001	2.664E-003	0.9821	0.9682	0.8765	26.775
RF	RQM	0.82	4	0.20	54.14	< 0.0001	0.11	0.9475	0.9300	0.8771	21.738

SS= Sum of Squares
DF= Degrees of freedom

MS= Mean of square

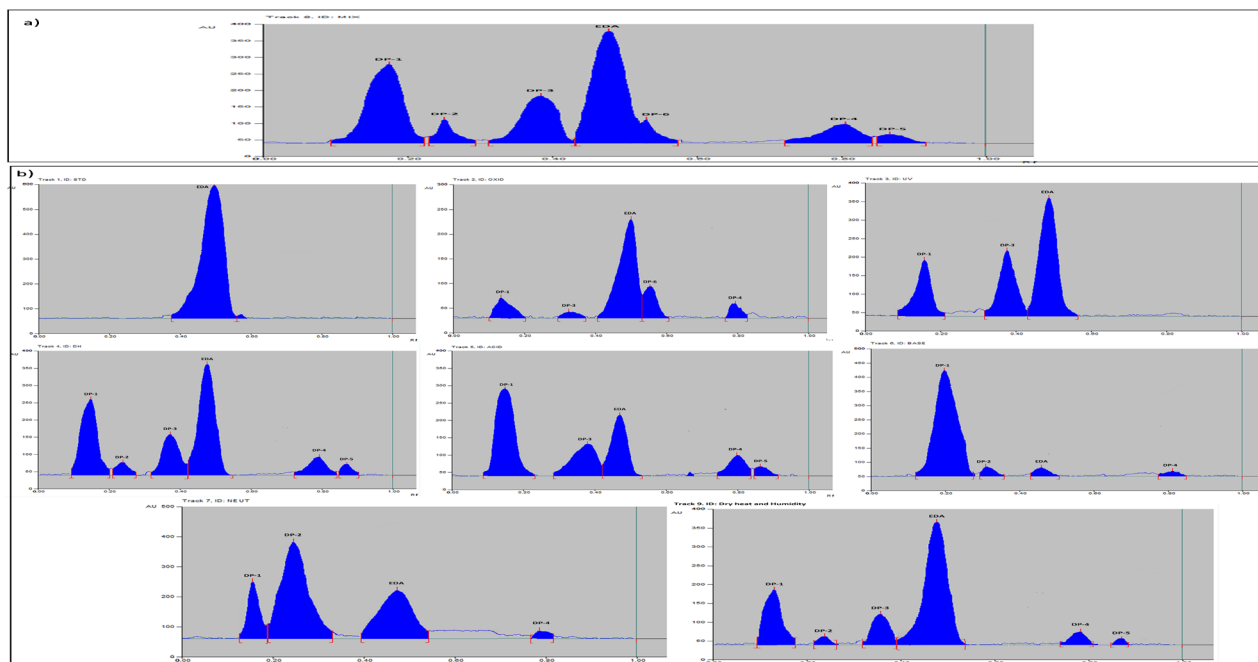
PRESS= prediction error sum of squares

Adj- r2= Adjusted r2

Pred- r2= Predicted r2

AP= Adequate Precision

RQM=reduced quadratic model



showed good resolution, selectivity, and symmetrical peaks and are shown in Figure 3a.

Method validation

Linearity and Range

The linearity of the method was investigated in the concentration range of 2- 24 µg/spot of EDA. The r^2 value was found to be 0.998. The overlay chromatogram and calibration curve is shown in Figure 4.

Precision

The % RSD of repeatability and reproducibility were found to be 0.742 and 0.957 respectively.

Limit of detection and quantitation (LOD and LOQ)

The LOD and LOQ of the present method were found to be 0.327 and 0.989 µg/spot respectively.

Specificity

The chromatogram of blank solution did not show any spot, while the chromatogram of the solution of the injection spiked with EDA gives clear and compact chromatogram of drug. No other peaks were eluted, therefore the method is considered to be specific.

Recovery studies

Standard addition method (corresponding to 80%, 100%, and 120%) was utilized to determine recovery of EDA from formulation matrix. The Values of recovery (%) and SD are found to be 100.27 ± 0.0503 , 99.88 ± 0.1123 and 99.93 ± 0.1113 respectively.

Robustness of the method

Robustness of the developed chromatographic method was determined by introducing small changes in the chamber saturation time, mobile phase composition, plate activation time, migration distance and volume of mobile phase. The effects on the results were examined that showed very slight changes in the peak area and Rf. The lower values %RSD indicates the robustness of method and shown in Table 3.

Stress degradation study

Stress degradation was carried out with bulk drug. Six DPs were formed in mixture of degradants (Figure 3a). The drug was found to be degraded under all stressed conditions. EDA was highly unstable when subjected to oxidation. The degradation conditions, % of degradation along with number of DPs formed are presented in Table 4. The chromatograms of individual stress degradations are shown in Figure 3b.

Application of developed method:

The proposed chromatographic method was used to estimate the content of EDA in commercially available

formulation. The method is selective for analysis of EDA, as no interference from the excipients was found. The proposed chromatographic method was utilized to analyze stability of EDA formulation. Stress degradation were carried out under same condition as specified for bulk drug and analyzed in the same way. The degradation products were well resolved with distinct Rf value. Minor variation was observed in % degradation of bulk drug and formulation (Table 4).

Degradation kinetic study

The degradation kinetic of EDA was investigated in acid, base, neutral and oxidative degradation. The linear regression analysis was performed for neutral and oxidative degradation, while acid and base degradation followed nonlinear regression analysis at the selected temperature and concentration.

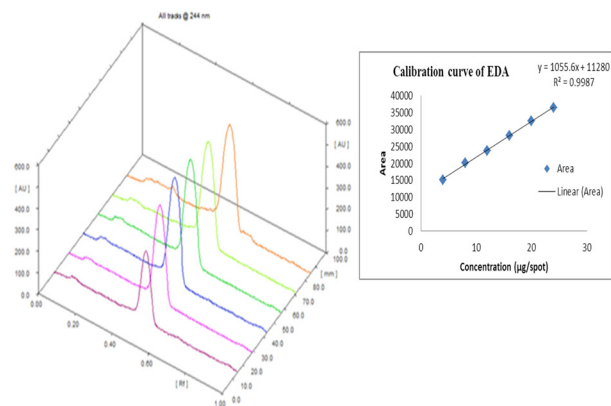


Figure 4: Overlay chromatogram showing linearity of developed method

Table 3: Results of Robustness study

Parameter	%RSD (Area)	%RSD (Rf)
Chamber Saturation Time		
20 min	0.44	0.66
25 min	0.56	0.75
Mobile Phase Composition Pet ether–Ethyl acetate–GAA		
6.0:4.0:10 µl, v/v/v/v	0.44	0.72
6.0:4.0:15 µl, v/v/v/v	0.38	0.69
5.6:4.4:10 µl, v/v/v/v	0.45	0.75
5.6:4.4:15 µl, v/v/v/v	0.74	0.99
Plate activation		
15 min	0.45	0.66
25 min	0.50	0.57
Volume of Mobile phase		
8 ml	0.24	0.54
12ml	0.41	0.66
Migration Distance		
88 mm	0.69	0.76
90 mm	0.51	0.77

Kinetics of neutral and oxidative degradation

Both neutral and oxidative degradation followed zero order degradation kinetic (Figure 5a and 5b) at selected concentration and temperature. The calculated rate constants were given in Table 5. Plot of % Degradation verses time gives the value of rate constant (estimated from slopes). The resulting K_{obs} values were plotted against temperature (in kelvin) to obtain Arrhenius plot which is presented in Fig. 5c for neutral degradation.

Kinetics of acid and base degradation

For both acid and base degradation nonlinear regression analysis (Figure 5d and 5e) was performed for pseudo

first order degradation kinetics at selected temperature and concentration as it fits better than linear regression analysis. Both acid and base degradation were assumed to follow pseudo first order degradation kinetic since the r^2 value is highest for second order process and the concentration of the stressor was much higher and constant during whole degradation process. The estimates of K_{obs} and $t_{1/2}$ (Table 5) can be obtained directly by plotting logarithm of observed % degradation vs time data in nonlinear regression analysis using graphpad prism. The Arrhenius plots are shown in Figure 5f and 5g for acid and base degradation respectively.

Table 4: Summary of stress degradation of EDA API and formulation

Stressor Type	Stressor Concentration	Time	% Degradation (API)	% Degradation (Formulation)	DPs Formed
Acid Degradation	0.05N HCl at 800C	180 m	63.51	61.24	DP-1, DP-3, DP-4, DP-5
Base Degradation	0.8N NaOH at 800C	180 m	69.34	67.09	DP-1, DP-2, DP-4
Neutral Degradation	100 0C	7 h	45.55	44.91	DP-1, DP-2, DP-4
Oxidative Degradation	6% H2O2 at RT	45 m	52.08	50.80	DP-1, DP-3, DP-4, DP-6
Photolytic Degradation	5382 LUX and 144UW/cm2	21 days	62.85	61.23	DP-1, DP-3
Dry Heat induced Degradation	80° C	21 days	67.42	20.41 (for 72 h)	DP-1, DP-3, DP-4, DP-5
Thermal/ humidity induced Degradation	40 ° C 70 + 5% RH	21 days	27.59	29.04	DP-1, DP-3, DP-4, DP-5

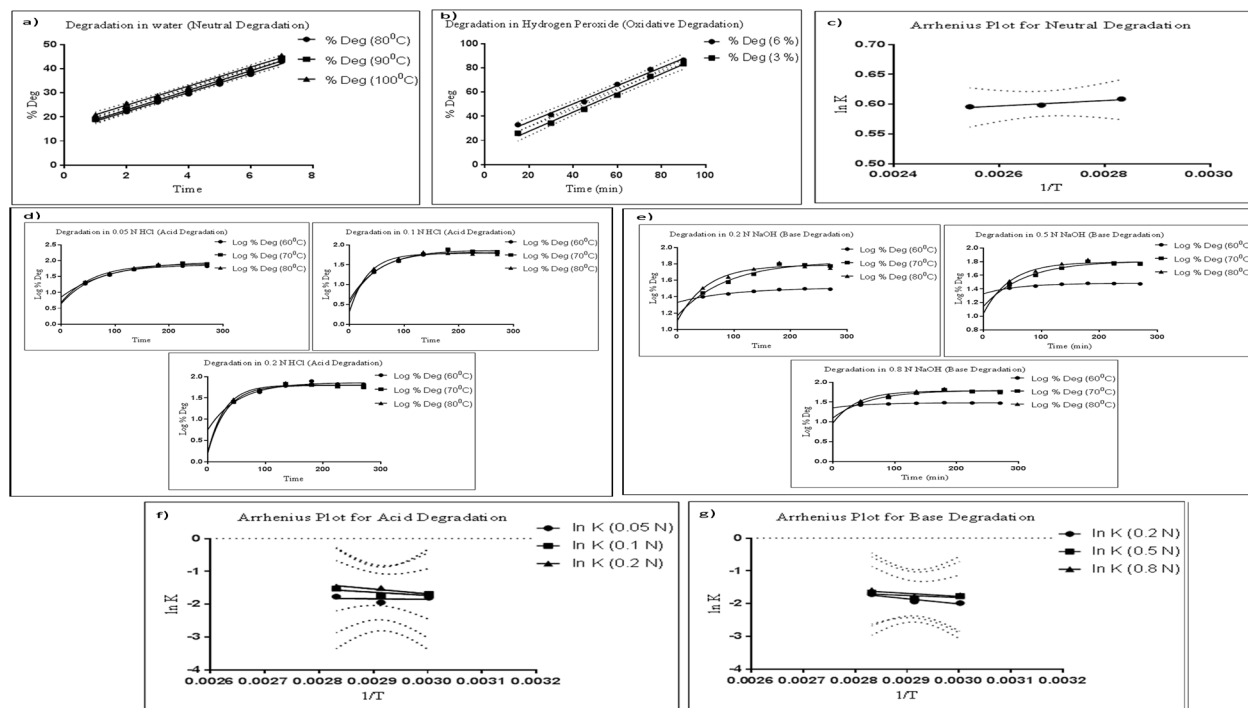


Figure 5: a) Linear zero order kinetic plot for neutral degradation, b) Linear zero order kinetic plot for oxidative degradation, c) Arrhenius plot for neutral degradation, d) Non-linear pseudo first order degradation kinetic plot for acid degradation, e) Non-linear pseudo first order degradation kinetic plot for base degradation, f) Arrhenius plot for acid degradation g) Arrhenius plot for base degradation.

Table 5: Reaction constants determined by linear and nonlinear regression analysis

Parameter	Experimental values								
Stressor	Neutral Degradation								
Temperature	(80°C)			(90°C)			(100°C)		
Concentration	--NA--			--NA--			--NA--		
r2	0.9957			0.9931			0.995		
Kobs	3.966			4.062			3.942		
t1/2 (min)	203.0594			198.2802			197.0606		
Ea	0.3797								
Stressor	Oxidative Degradation								
Temperature	--NA--			--NA--			--		
Concentration	6 %			3 %			--		
r2	0.9928			0.9933			--		
Kobs	0.7504			0.7915			--		
t1/2 (min)	--			--			--		
Ea	--								
Stressor	Acid Degradation								
temperature	(60°C)			(70°C)			(80°C)		
concentration	0.05 N	0.1 N	0.2 N	0.05 N	0.1 N	0.2 N	0.5 N	0.1 N	0.2 N
R2	0.9977	0.9892	0.9873	0.9989	0.974	0.9793	0.9958	0.958	0.9519
Kobs	0.0159	0.0205	0.0193	0.0114	0.0182	0.0314	0.0170	0.0300	0.0345
t1/2 (min)	43.49	33.76	35.79	61.05	38.05	22.01	40.72	23.05	20.09
Ea	-1.20			-7.94			-12.37		
Strssor	Base Degradation								
temperature	(60°C)			(70°C)			(80°C)		
concentration	0.2 N	0.5 N	0.8 N	0.2 N	0.5 N	0.8 N	0.2 N	0.5 N	0.8 N
R2	0.9847	0.9682	0.9439	0.9818	0.9825	0.9778	0.964	0.9623	0.9289
Kobs	0.0112	0.0174	0.0184	0.0147	0.0172	0.0198	0.0219	0.0244	0.0283
t1/2 (min)	66.44	41.07	38.39	59.76	48.92	41.26	35.67	31.79	26.77
Ea	-13.10			-5.27			-7.53		

CONCLUSION

As there is no reported stability indicating TLC-densitometry method for edaravone, the goal of present work was achieved by implementing the risk based and systematic QbD approach to resolve and quantitate Edaravone in presence of its degradation products in bulk and injection formulation. The developed TLC-densitometry method has been validated as per ICH guidelines for the determination of the drug without any interference from excipients and in presence of degradation products. The method provides significant sensitivity as well as reduced sample preparation and instrument run time over other separation methods. Also the method offers advantages such as short system equilibrium time, possibility of simultaneous analysis of samples and standard on the same plate, higher mobile phase pH and minimum solution consumption. Hence the method appears to be suitable for routine analysis

and for quality control in the pharmaceutical industry due to its simplicity, sensitivity and selectivity.

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CONFLICT OF INTEREST

None

ABBREVIATION USED

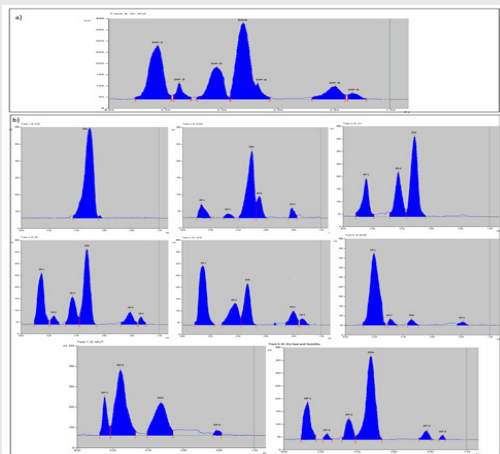
EDA: Edaravone; HCl: Hydrochloric acid; NaOH: Sodium hydroxide; H₂O₂: Hydrogen peroxide; QbD: Quality by design; ATP: Analytical target profile; BBD: Box behenkan design; ICH: International conference of harmonization; Deg: Degradation; MPC:mobile phase composition; CST: chamber saturation time; MD:

migration distance; Rf: Retardation factor; LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation.

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PICTORIAL ABSTRACT



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SUMMARY

- Stability indicating QbD based TLC-densitometry method was developed and validated as per ICH guidelines. Edaravone was subjected to ICH prescribed stress degradation conditions and was found to be susceptible under all conditions. The Rf value of Edaravone was 0.46 ± 0.21 and the method was found to be linear over the range of 04-24 $\mu\text{g}/\text{band}$. The LOD and LOQ were found to be 0.327 and 0.989 $\mu\text{g}/\text{spot}$ respectively. Linear and non-linear regression analysis was performed to determine degradation kinetic. The kinetics of acid and base degradation followed non-linear kinetics while neutral and oxidative degradation followed linear degradation kinetic.

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