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OPTIMIZATION OF A FORCED DEGRADATION STUDY OF ATORVASTATIN EMPLOYING AN EXPERIMENTAL DESIGN APPROACH

Maja Hadzieva Gigovska[1*], Ana Petkovska[1], Jelena Acevska[2], Natalija Nakov[2], Blagica Manchevska[1], Packa Antovska[1], Sonja Ugarkovic[1], Aneta Dimitrovska[2]

[1]*Research & Development, ALKALOID AD, Blvd. Aleksandar Makedonski 12, 1000 Skopje, Republic of Macedonia*
[2]*Faculty of Pharmacy, University Ss. Cyril and Methodius, Mother Theresa 47, 1000 Skopje, Republic of Macedonia*

hadzievam@yahoo.com

This study involved the optimization of experimental conditions for the forced degradation of atorvastatin employing the experimental design (DoE) approach, as a scientific multifactorial strategy. Using 2^n full factorial design, stress conditions of oxidative, hydrolytic and thermal degradation were optimized to obtain a targeted level of atorvastatin degradation. Atorvastatin and all related and degradation products were separated on Poroshell 120 EC C18 50 × 3.0 mm 2.7 μm, using 10 mM ammonium formate and acetonitrile as mobile phases in the gradient mode. The impurity structures were confirmed by the direct hyphenation of a liquid chromatograph to an ion trap mass spectrometer with a heated electrospray ionization interface.

This study highlights the multifold benefits of implementing the DoE concept, which provides a better understanding of the significant factors responsible for degradation and ensures a successful way to achieve degradation, thereby replacing the trial and error approach used in conventional forced degradation studies.

Keywords: atorvastatin; related and degradation products; LC/MS; experimental design; forced degradation

ОПТИМИЗАЦИЈА НА ЕКСПЕРИМЕНТАЛНИТЕ УСЛОВИ ЗА ФОРСИРАНА ДЕГРАДАЦИЈА НА АТОРВАСТАТИН КОРИСТЕЈЌИ ЕКСПЕРИМЕНТАЛЕН ДИЗАЈН

Оваа студија опфаќа оптимизација на експериментални услови за форсирана деградација на аторвастатин, користејќи експериментален дизајн (DoE), како научна мултифакторна стратегија. Користејќи 2^n полн факторски дизајн, беа оптимизирани стрес-условите на оксидативна, хидролитичка и термичка деградација за да се постигне саканото ниво на деградација на аторвастатин. Оптимално разделување на аторвастатин од неговите нечистотии беше постигнато со градиентно елуирање, користејќи 10 mM амониум формат и ацетонитрил како мобилна фаза на Poroshell 120 EC C18 50 × 3,0 mm 2,7 μm.

За идентификација и потврда на структурата на нечистотиите беше користена течна хроматографија под висок притисок (HPLC) поврзана со масена спектрометрија (MS) со електроспреј-јонизација (ESI).

Оваа студија ги истакнува повеќекратните придобивки од спроведувањето на концептот DoE, кој обезбедува подобро разбирање на значајните фактори одговорни за деградацијата и обезбедува задоволителен степен на деградација. Овој пристап може да го замени конвенционалниот пристап на проба и грешка кој се користи во студиите на форсирана деградација.

Клучни зборови: аторвастатин; сродни и деградациони продукти; LC/MS; експериментален дизајн; форсирана деградација

1. INTRODUCTION

Forced degradation studies provide data to support the identification of possible degradants, degradation pathways and the intrinsic stability of the drug molecule, as well as validation of the stability indicating analytical procedures [1]. Although most of the literature defines the concept of forced degradation, detailed information about a forced degradation strategy is not provided [2]. The experimental conditions to conduct forced degradation are described in a general way without a description of the exact stress conditions to be applied [3–8].

Generally, a trial and error approach are adopted to select the strength, temperature and time of exposure to achieve a loss of active substance from 5–20 % [3, 9–11]. Due to the considerable cost, time consumption, scientific expertise and high incidence of random results, the need for a more systematic approach is recognized.

A contemporary approach in the field of forced degradation is to evaluate the correlation of degradation parameters by applying the experimental design approach (DoE). In turn, this approach allows the combination of conditions where optimal degradation is obtained to be studied.

The basic concept of factorial design is performing an experiment in which all possible combinations of factors and levels are investigated [12]. This design determines the effect of each factor on the response as well as how the effect of each factor varies with the change in level of other factors. The advantages of factorial designs over one-factor-at-a-time experiments are that they are more efficient and allow interactions to be detected [12].

The literature review reveals the implementation of DoE for the optimization of forced degradation conditions, but no report exists on the application of the DoE concept for the forced degradation of atorvastatin as a model drug [13–16].

Atorvastatin is a synthetic competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A, that has been confirmed to be efficacious in reducing both cholesterol and triglyceride [17]. It is used in the treatment of hypercholesterolemia and dyslipidemia [17]. Chemically, atorvastatin is (3*R*,5*R*)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-yl-pyrrol-1-yl]-3,5-dihydroxyheptanoic acid [17]. The literature data have shown that atorvastatin, like most of the statins, is a very unstable molecule and the analytical processing of this active ingredient is still a current problem. Also, reviewed literature data include only a few studies, where the instability of atorvastatin in hydrolytic, oxidative or photolytic condition was mentioned as a part of the selectivity of the stability-indicating methods [18–30]. To date, to the best of our knowledge, none of the reported analytical procedures describe a simple and satisfactory sample preparation methodology where the influence of the stressor straight, time of expose and temperature are evaluated in detail.

Therefore, the goals of the present study are to explore the degradation behavior of atorvastatin under different stress conditions and to simplify forced degradation studies by adopting a DoE approach. In order to resolve all of the possible degradation products, a method compatible with mass spectrometry was developed which will be used as confirmation to the results obtained with the compendia method.

2. EXPERIMENTAL

2.1. *Chemicals and standards*

Atorvastatin calcium trihydrate CRS (purity 95.2 %), Atorvastatin Impurity A, Atorvastatin Impurity B, Atorvastatin Impurity C, and Atorvastatin Impurity D were provided by European Directorate for the Quality of Medicines and Health Care Council of Europe (EDQM–Strasbourg, France). Atorvastatin Impurity H (Atorvastatin lactone) produced by LGC Im Biotechnologiepark-TGZ II D-14943 Luckenwalde, Germany, was used.

Atorvastatin active substance samples with certificates of suitability to the monographs of the European Pharmacopeia (CEP), received as free samples from MSN Pharmachem Pvt. Ltd., India, were used.

Analytical grade acetonitrile, ammonium formate, formic acid, ammonium acetate, tetrahydrofuran, glacial acetic acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck (Darmstadt, Germany).

Water was purified by a Werner water purification system, obtained in-house at Alkaloid AD Skopje, Skopje, Macedonia.

Regenerated cellulose membrane syringe filters with pore size 0.45μm, were purchased from Phenomenex (Torrance, CA. USA).

2.2. *Experimental conditions*

2.2.1. *High performance liquid chromatography*

Experiments were performed on Agilent Technologies 1260 Series Quaternary Liquid Chromatographic System (Agilent Technologies, USA) equipped with a Quaternary Pump (G13112B), a column compartment (G1316A), thermostat (G1330B), auto sampler (G1367E) and photodiode array detector (G4212B). Instrument control, data acquisition and processing were performed by using OpenLab Chemstation chromatography software (version A.02.02/1.3.4).

The quantification of atorvastatin and its related degradation products was performed according to the method presented in the European Pharmacopeia monograph using Zorbax SB C8 (Agilent Technologies, USA) 250 × 4.6 mm, 5 μm particle size [28].

2.2.2. *Liquid chromatography–tandem mass spectrometry (LC-MS)*

The LC–MS/MS analyses were conducted on Dionex UltiMate™ 3000 UHPLC-UV-DAD (Thermo Fisher Scientific, Waltham, MA, USA), interfaced with a linear ion-trap mass spectrometer (LTQ XL) equipped with a heated electrospray ionization source operating in the positive ionization mode. Instrument control and results processing was done using Dionex Chromeleon 7.2 (for HPLC-DAD analyses) and Thermo Xcalibur v2.2 SP1 (for HPLC-DAD/MS analyses). Structural confirmation and fragment elucidation was performed using Mass Frontier v7.0.

The separation was performed on Poroshell 120 EC C18 (Agilent Technologies, USA), 50 × 3.0 mm, 2.7 μm using buffer (10 mM ammonium format, pH 4.0) and acetonitrile as a mobile phase in a gradient mode as follows: T(min)/Acetonitrile (%) 0/30; 3.5/30; 7/35; 20/65; 25/66; 40/30. The column temperature was 40 °C. Flow rate was 0.5 ml/min. Injection volume was 5 μl. UV detection was performed at 238 nm.

Mass parameters were optimized as follows: ion source heater temperature was set at 280 °C and capillary temperature at 200 °C; capillary voltage was 20 V with collision energy 35 eV.

Nitrogen was used as a nebulizing gas at a pressure of 50 psi and the flow was adjusted to 10 l/min. MS data were acquired in the negative ionization mode. The full scan covered the mass range at *m/z* 100–1200. Collision–induced fragmentation experiments were performed in the ion trap using helium as a collision gas, with a voltage ramping cycle from 0.3 up to 2 V. The maximum accumulation time of ion trap and the number of MS repetitions to obtain the MS average spectra were set at 500 ms and 3 ms, respectively.

As can been seen from above, two independent HPLC methods with UV (compendia method described in European Pharmacopeia) and MS detection were used to monitor degradation and to support the peak identification procedure.

2.3. *Standard and sample preparation*

2.3.1. *Standard preparation*

A mixture of atorvastatin at a concentration of 0.05 mg/ml spiked with impurities (namely Atorvastatin Impurity A, B, C and D in concentration of 0.05 mg/ml each) was used as a system suitability solution. The standard solution of atorvastatin in a final concentration of 0.001 mg/ml was used for the quantitative determination of unknown compounds, corresponding to a 0.1 % of concentration of the main compound in the sample solution. A mixture of acetonitrile and water at a ratio of 50:50 (*v/v*) was used as a diluent.

2.3.2. *Sample preparation*

Atorvastatin was subjected to stress under acidic, alkaline, oxidative, thermal and photolytic conditions.

In the preliminary experiments, atorvastatin was subjected to 0.1 N HCI for one hour and 0.5 N NaOH for 48 h at ambient temperature (25 ± 2 °C). The oxidation stress was performed with a 3 % H_2O_2 solution for 48 h at ambient temperature (25 ± 2 °C). For thermal degradation, atorvastatin was exposed at 60 °C for 10 days.

A photodegradation study was performed by exposing the drug powder, spread as a thin film in Petri plates and exposed to direct sunlight for one and two days. Additionally, a control study in the dark was run simultaneously.

2.3.2.1. *Sample preparation according to full factorial design for acid, alkali, oxidative and thermal degradation*

The forced degradation experiments set-up on the basis of 2^n full factorial design were per-

formed and the obtained results were analyzed by Design Expert Software (Stat-Ease Inc. Minneapolis, MN. USA). Acid, alkali and oxidative degradation were performed using 2^3 factorial design (three variables: stressor strength, temperature and time considered at two levels) as presented in Table 1.

T a b l e 1

Experimental conditions and results from 2^3 full factorial design for acid, alkali and oxidative degradation

Experimental conditions				Responses											
Factor levels [a]				Acid degradation				Alkali degradation				Oxidative degradation			
Exp. No	x_1	x_2	x_3	y_1	y_2	y_3	y_4	y_1	y_2	y_3	y_4	y_1	y_2	y_3	y_4
1.	–	–	–	2.84	2.79	2.27	0.04	0.15	0.70	0.04	0.09	3.42	5.34	0.60	0.77
2.	+	–	–	11.25	11.05	10.98	0.05	0.53	0.73	0.02	0.12	13.51	13.09	1.55	7.62
3.	–	+	–	2.85	2.80	2.35	0.05	0.22	0.72	0.06	0.10	17.48	17.14	0.98	5.83
4.	+	+	–	8.23	8.08	7.55	0.08	0.33	0.93	0.12	0.12	46.28	45.35	0.25	13.85
5.	–	–	+	12.79	12.56	11.83	0.06	0.28	0.85	0.26	0.12	8.51	8.35	0.45	5.47
6.	+	–	+	19.05	19.01	18.40	0.09	0.36	0.89	0.25	0.12	16.97	16.64	2.10	9.23
7.	–	+	+	15.84	15.56	13.97	0.05	0.24	0.64	0.10	0.14	22.65	22.24	1.02	7.77
8.	+	+	+	19.48	19.14	18.48	0.05	0.23	0.69	0.13	0.10	43.32	42.27	0.82	23.78

[a] Aberrations
x_1: Stressor strength 0.1 M and 1 M HCl / NaOH 3 % and 30 % H_2O_2
x_2: Temperature 25 °C and 60 °C
x_3: Time 15 and 60 minutes
y_1: amount of Total Impurities (%) determinate by LC/MS method
y_2: amount of Total Impurities (%) determinate by European Pharmacopeia method
y_3: amount of Atorvastatin Impurity H (%) determinate by European Pharmacopeia method
y_4: amount of Unknown Impurity (%) determinate by European Pharmacopeia method
The high level of each factor was considered as "+" and low level as "−".

A set of eight experiments was performed, as stated in Table 1. For acid degradation, 2 ml of atorvastatin stock solution (10.0 mg/ml) was treated with 2.0 ml x_1 hydrochloric acid (M HCl) heated at 25 °C and 60 °C for 15 and 60 minutes. At the end of exposure, the samples were neutralized with 2.0 ml sodium hydroxide (0.1 M or 1 M NaOH, respectively) and diluted to a final concentration of 1 mg/ml with diluent.

The study in alkaline conditions was performed in a similar manner according to the experimental plan presented in Table 1 using 0.1 and 1 M sodium hydroxide solution, neutralized with 2.0 ml hydrochloric acid solution (0.1M or 1M HCl, respectively) and diluted to a final concentration of 1 mg/ml.

For oxidative degradation, three variables were considered at two levels (the high level for H_2O_2, temperature and time of exposure were 30 %, 60 °C and 60 minutes, and the low level were 3 %, 25 °C and 15 minutes, respectively). The peroxide reactions at a higher temperature (60 °C) were carried out by taking adequate precautions.

The 2^2 factorial designs were conducted to set up thermal degradation, where the high-level values were 105 °C and 5 h and the low levels were 80 °C and 3 h, respectively, as shown in Table 2.

All stress studies were performed in amber glassware, in order to protect the solutions from light degradation, and filtered through a 0.2 μm regenerated cellulose membrane filter.

Several control samples were prepared for comparison with the stressed samples. Blank solutions consisting of stress agents were treated and analyzed in the same manner to mark the peaks corresponding to stress agents and to distinguish them from the potential degradation products. Additionally, the drug solution stored under normal conditions was analyzed.

T a b l e 2

Experimental conditions and results from 2^2 full factorial design for thermal degradation

Number of experiments	Factor levels [b]		Responses			
	x_1	x_2	y_1	y_2	y_3	y_4
1	–	–	0.76	1.73	0.58	0.10
2	–	+	0.74	1.79	0.61	0.09
3	+	–	1.13	2.41	0.83	0.08
4	+	+	1.24	2.56	0.92	0.09

[b] Aberrations
x_1: Temperature 80 °C and 105 °C
x_2: Time 3 and 5 h
y_1: amount of Total Impurities (%) determinate by LC/MS method
y_2: amount of Total Impurities (%) determinate by European Pharmacopeia method
y_3: amount of Atorvastatin Impurity H (%) determinate by European Pharmacopeia method
y_4: amount of Unknown Impurity (%) determinate by European Pharmacopeia method

3. RESULTS AND DISCUSSION

3.1. *Lability of the drug under different forced degradation conditions*

According to the results obtained from our preliminary experiments, described in Section 2.3.2, atorvastatin is susceptible to degradation under oxidative and acid hydrolyses, and is slightly degraded under UV light and alkali hydrolysis. Also, atorvastatin was found to be stable to thermal degradation. The obtained results are presented in Figure 1.

In the preliminary experiments, it was found that Atorvastatin Impurity A remained unaffected by all of the stress conditions applied, and only slightly increased after light exposure. Atorvastatin Impurity B and C were not detected at all. Atorvastatin Impurity D is proven to be an oxidative degradation product and arises at a higher level after exposure to H_2O_2, but the maximal obtained degradation was 0.11 %. Atorvastatin Impurity H was found to be sensitive to all stress conditions applied, especially after acid hydrolysis. Therefore, besides the percentage of total impurities, the amount of Atorvastatin Impurity H formed was chosen to be evaluated with the proposed experimental design approach.

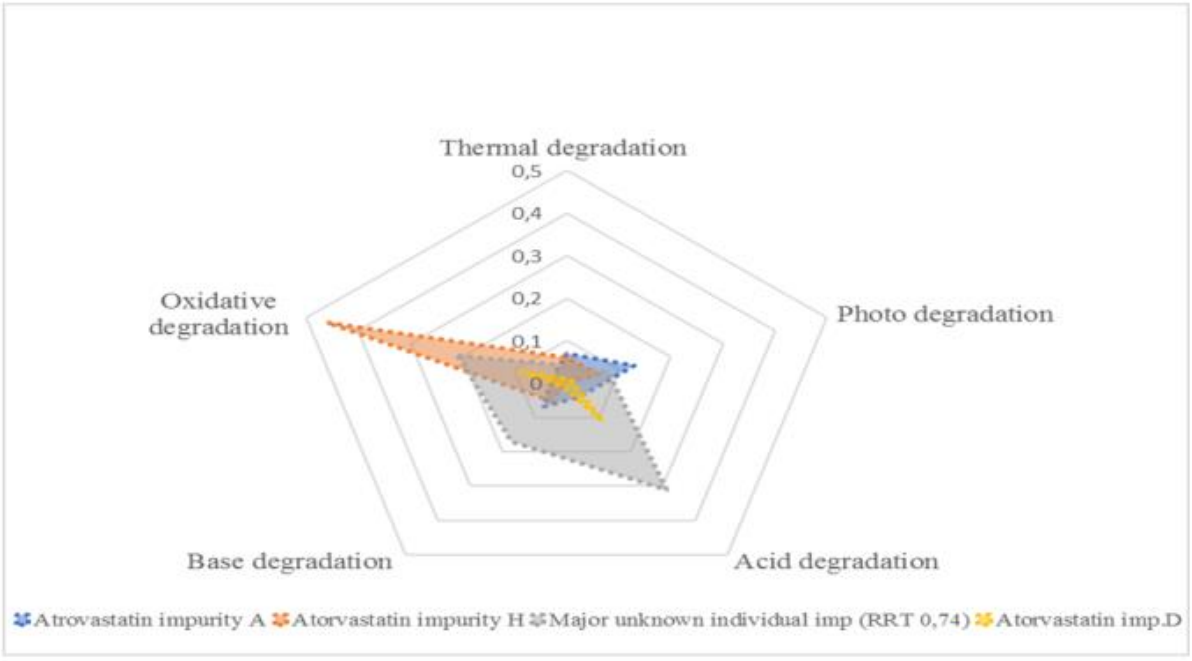


Fig. 1. Graphical presentation of the obtained results from preliminary degradation study

3.2. *Optimization of various forced degradation conditions by the experimental design approach*

Experimental design is a powerful analytical tool to systematically investigate the influence of each factor and to find the optimum parameter settings in order to obtain the desired values of responses. The application of an experimental design may contribute to a good relation of cost and benefits and also allows the most optimal conditions to be estimated in order to assure the quality of the conducted experiments.

General steps involved in the experimental design strategy were the selection of variables, response, adequate design and defining an experimental plan. Screening of the important and most affecting variables was performed by using one-way ANOVA and Pareto chart; the selection of the optimum region was made by evaluating the 3D response surface plots.

The preliminary experiments and detailed literature survey presented in Table 3 provided valuable information about the experimental region and the definition of factor intervals. Stressor strength, time of exposure and temperature were identified as factors which should be analyzed.

The selection of the type and concentration of hydrogen peroxide, acid or base was made by taking into consideration the results from our previous preliminary experiments and the detailed literature review shown in Table 3 [1, 3, 5].

Hydrochloric acid and sodium hydroxide at concentrations of 0.1 M and 1 M were evaluated as suitable reagents for hydrolysis. The concentrations of hydrogen peroxide of 3 % and 30 % were used for oxidative forced degradation.

The effect of temperature on acid, alkali and oxidative degradation of atorvastatin was studied at two levels: 25 °C and 60°C, respectively. Hydrolytic degradation is usually performed at room temperature, and if no degradation is observed at room temperature, then the temperature can be increased. When implementing the experimental design, the simultaneous evaluation of the effect of temperature was performed in just a few experiments.

The time of exposure was chosen based on the minimum length in order to gain information about degradation over a short time; therefore, the low and high levels were set to 15 minutes and 60 minutes, respectively.

Thermal degradation studies were performed at 80 °C following the general recommendation stated in the ICH guidelines [9]. Therefore, 80 °C was considered the low level and 105 °C was considered the high level. The time of exposure was chosen to be 3 and 5 hours.

T a b l e 3

Summary of literature information about conditions for conducting forced degradation studies of atorvastatin

Acid degradation			Alkali degradation			Oxidative degradation			Thermal degradation		Reference
Strength HCL	Time	T °C	Strength NaOH	Time	T °C	Strength H_2O_2	Time	T °C	Time	T °C	
0.1M	1 h	40 °C	0.1 M	1 h	40 °C	3 %	1 h	40 °C	2 h	80 °C	[18]
0.1M	6 h	22 °C	0.1 M	6 h	22 °C	3 %	6 h	22 °C	3 h	80 °C	[19]
0.1M	24 h	/	0.1 M	24 h	/	3 %	24 h	/	24 h	50 °C	[20]
/	/	/	/	/	/	30 %**	5 h	55 °C	/	/	[22]
1M	24 h	/	1 M	24 h	/	3 %	24 h	/	24 h	60 °C	[21]
1M	4 h	/	1 M	4 h	/	3 %	4 h	/	4 h	BWB*	[23]
0.1M	45 min	BWB*	0.1 M	45 min	BWB*	3 %	45 min	BWB*	45 min	BWB*	[24]
1M	1 h	/	1 M	1 h	/	30 %	1 h	/	2 h	80 °C	[25]
1M	30 min	BWB*	1 M	30 min	BWB*	3 %	30 min	BWB*	45 min	BWB*	[27]
0.1M		80 °C	1 M		80 °C	30 %		RT	15 days	50 °C	[29]
0.1M	24 h	25 °C	1 M	42 h	25 °C	1 %	24 h	25 °C	10 days	105 °C	[30]

Aberrations BWB * Boiling water bath

** 30 % H_2O_2 +12MNaOH

The regulatory guidance does not specify the initial concentration of a compound for forced degradation studies [9]. The initial concentration recommended in several studies ranged from 0.1–1 mg/ml [1, 3]. Also, the concentration stated in the official monograph in the European Pharmacopeia is 1 mg/ml [28]. In order to get even minor decomposition products in the range of detection and to overcome the need for additional validation of the method, an initial concentration of 1 mg/ml was chosen for this study.

In this study, the amount of Total Impurities (%), Atorvastatin Impurity H and Unknown Impurity were chosen as dependent variables.

All samples were simultaneously evaluated with two independent methods. A mass spectrometry compatible HPLC method, as described in Section 2.2.2, was used to detect impurities and as a confirmation of the results obtained with the compendia method. A parallel comparison in the amounts determined by a compendia HPLC method and proposed LC/MS method was made. The obtained results are presented in Table 1 and Table 2, respectively. As can be seen, the amounts determined by the two methods were in agreement with a margin of analytical error. Therefore, the interpretation of the results discussed below is valid for both methods.

3.2.1. *The model equations and statistical evaluation*

The results obtained for each type of forced degradation (analyzed with compendia method) like the percentage of Total Impurities were considered as the dependent variable (y_1) and were subjected to multiple regression analysis. The following type of equation was obtained:

$$y = b_0 + b_1A + b_2B + b_3C + b_{12}AB + b_{13}AC + b_{23}BC + b_{123}ABC$$

where b_0 is the intercept b_1, b_2 and b_3, b_{12}, b_{23}, b_{12} and b_{123} as regression coefficients for the variables and interaction between the variables. The obtained results for the regression coefficients are presented in Table 4.

The adequacy of the proposed design was statistically assessed by several statistical criteria, such as coefficient of determination (R^2), Adjusted R^2, Predicted R^2 and Adequate Precision. As can be seen in Table 4, the calculated values of the R-Squared value are in the range from 0.91–0.99, which indicates that about 91–99% of the data variability was successfully explained by the model in all cases. In this study, the obtained values of Adjusted R^2 were well within the agreeable bounds of R^2, which revealed that the experimental data are a good fit with the calculated equations. In addition, the predicted R^2 in all conditions is in reasonable agreement with the adjusted R^2 (within 0.2), which also implies the good predictability of models, except in alkali degradation. For alkali degradation, the predicted R^2 of –0.347 is not as close to the adjusted R^2 of 0.7142 as one might normally expect. According to the literature data, atorvastatin is slightly degraded under alkali hydrolysis and the obtained results correlated to previously known degradation behavior [30]. Evaluation of the results shows an adequate value for "Adeq. Precision", a parameter which measures the signal to noise ratio (5.908); this model can be used to navigate the design space.

T a b l e 4

The regression coefficients obtained for % Total Impurities, % Atorvastatin Impurity H, % Unknown Impurity

% Total Impurities	Regression coefficients	R^2	R^2 Predicted	R^2 Adjusted
Acid degradation	$b_0 = 11.37$; $b_1 = 2.95$; $b_2 = 0.021$; $b_3 = 5.19$; $b_{12} = –0.73$; $b_{23} = 0.76$	0.9946	0.9166	0.9816
Alkali degradation	$b_0 = 0.77$; $b_1 = 0.041$; $b_2 = –0.024$; $b_3 = –0.00124$; $b_{12} = 0.024$; $b_{23} = – 0.079$	0.9183	–0.3471	0.7142
Oxidative degradation	$b_0 = 21.33$; $b_1 = 8.06$; $b_2 = 10.48$; $b_3 = 1.09$; $b_{12} = 4.05$	0.9881	0.9155	0.9723
Thermal degradation	$b_0 = 2.13$; $b_2 = 0.37$	0.9708	0.9562	0.8832
% Atorvastatin Impurity H				
Oxidative degradation	$b_0 = 0.97$; $b_1 = 0.21$; $b_2 = – 0.21$; $b_3 = 0.12$; $b_{12} = –0.44$; $b_{13} = 0.15$	0.9965	0.9877	0.9432
% Unknown Impurity				
Oxidative degradation (Square root of % degradation impurity)	$b_0 = 2.85$; $b_1 = 0.75$; $b_2 = 0.60$; $b_3 = 0.41$; $b_{123} = 0.25$	0.9362	0.8884	0.7449
Aberration A: Stressor strength HCl / NaOH or H_2O_2; B: Temperature; C: Time of exposure				

The significance test for the model fit was evaluated by ANOVA in order to determine the significant and most contributing factors which were ranked on the basis of the degree of F-ratio. Higher F-values correspond to smaller "Prob>F" values. Table 5 shows the reading of the ANOVA analysis where the F-value and P-value of the model were 76.33 and 0.0130, respectively, for acid degradation, demonstrating that the estimated model fits the experimental data satisfactorily. Additionally, results for ANOVA for all degradation conditions are presented in the same manner in Table 5. They indicate that the chosen model for all of the investigated conditions fit the experimental data satisfactorily.

Next, the qualitative contribution of each factor and respective responses were analyzed for all various conditions of degradation. Each response coefficient was studied for its statistical significance by Pareto charts, as shown in Figure 2. Pareto charts establish the t value of the effect by two limit lines, namely the Bonferroni limit line and the *t* limit line. Coefficients with a *t* value of effect above the Bonferroni line are designated as certainly significant. Coefficients with a *t* value of effect between the Bonferroni line and the *t* limit line are termed as coefficients likely to be significant, while a *t* value of effect below the *t* limit line is statistically insignificant.

Pareto chart analyses indicated that the strength of hydrochloric acid and time of exposure were the most significant factors under acidic conditions (Fig. 2A).

For alkali hydrolysis, all of the investigated factors have t values of effect below the t limit line and are statistically insignificant within the range evaluated, at the 95 % confidence interval (Fig. 2B), proving the stability of the drug in the investigated conditions.

T a b l e 5

ANOVA for 2^n full factorial design for different type of degradation evaluating the % of Total Impurities

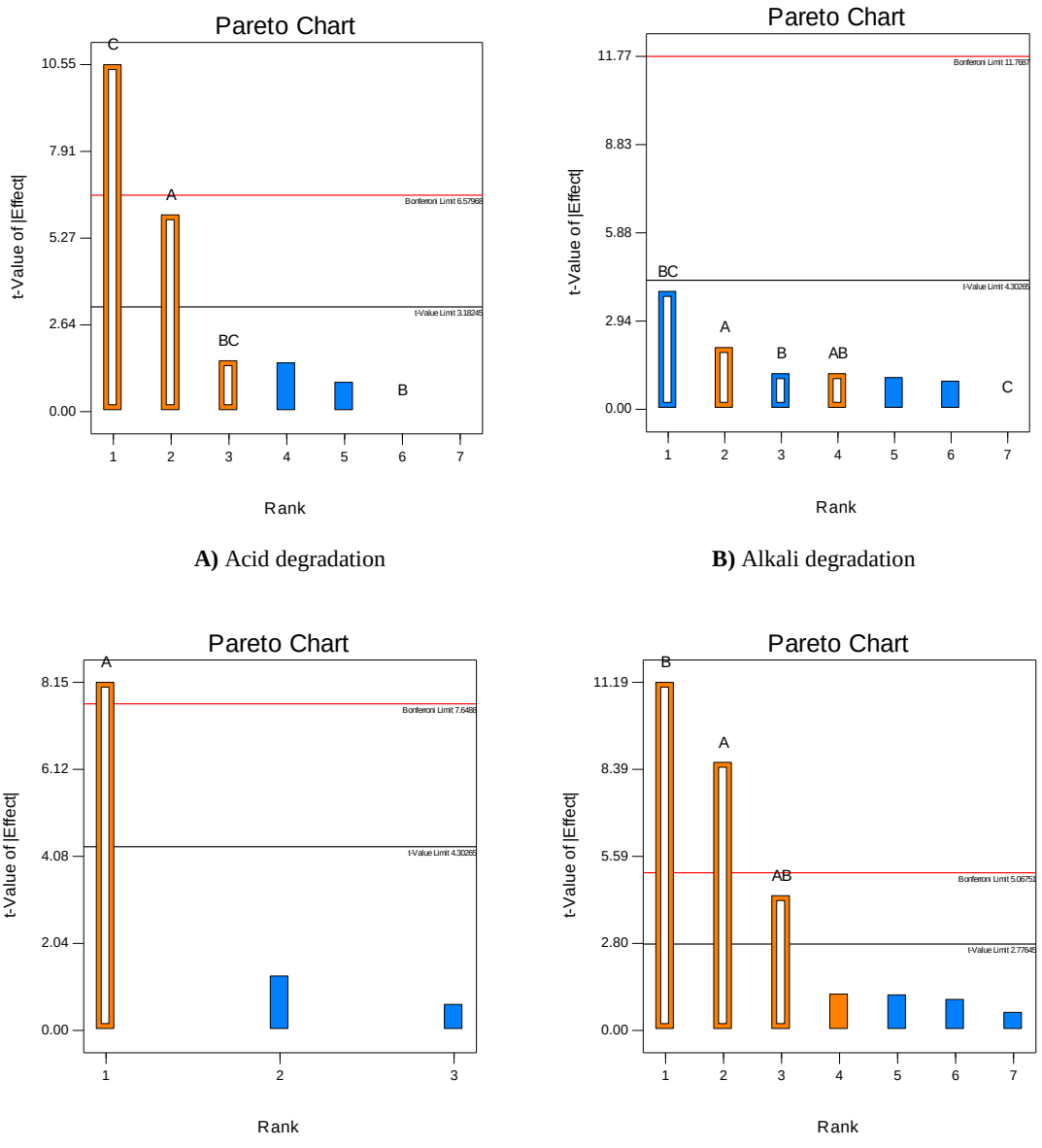
	Sum of squares	df	Mean square	F-value	P-value Prob > F	
Acid degradation						
Model	294.16	5	58.83	76.33	0.0130	significant
A–Strength HCl	69.44	1	69.44	90.10	0.0109	
B–Temperature	3.613E-003	1	3.613E-003	4.68E-003	0.9516	
C–Time	215.80	1	215.80	279.98	0.0036	
*AB–Strength HCl*Temperature*	4.28	1	4.28	5.55	0.1426	
BC–*Temperature*Time*	4.64	1	4.64	6.01	0.1337	
Residual	1.54	2	0.77			
Cor Total	295.70	7				
Alkali degradation						
Model	0.072	5	0.014	4.50	0.1918	Not significant
A–Strength NaOH	0.014	1	0.01	4.24	0.1758	
B–Temperature	*4.513E-003*	1	0.0045125	1.40	0.3577	
C–Time	*1.250E-005*	1	1.25E-05	0.00	0.9559	
*AB–Strength NaOH*Temperature*	*4.513E-003*	1	0.0045125	1.40	0.3577	
*AC–Strength NaOH*Time*	0.05	1	0.05	15.44	0.0591	
Residual	6.425E-003	2	3.213E-003			
Cor Total	0.079	7				
Thermal degradation						
Model	0.54	1	0.54	66.49	0.0147	significant
A–Temperature	0.54	1	0.54	66.49	0.0147	
Residual	0.02	2	8.125E-003			
Cor Total	0.56	3				
Oxidative degradation						
Model	1539.24	4	384.81	62.40	0.0032	significant
A–*Strength* H_2O_2	520.19	1	520.19	84.36	0.0027	
B–*Temperature*	878.01	1	878.01	142.38	0.0013	
C–*Time*	9.57	1	9.57	1.55	0.3013	
AB–*Strength* H_2O_2**Temperature*	131.46	1	131.46	21.32	0.0191	
Residual	18.50	3	6.17			

Aberrations:
df: degree of freedom. Cor. Total: corrected total sum of squares. Values of Prob > F less than 0.05 indicates models are statistically significant

As could be expected in thermal degradation, temperature has the biggest impact (Fig. 2C). This kind of effect was expected because it is known that kinetic constants have exponential dependency with reaction temperature (Arrhenius law) and this has also been reported by other authors [22]. Therefore, in this case, further investigations were not performed. The obtained results imply confirmation of the well-known behavior of atorvastatin at elevated temperatures.

For oxidative degradation, the strength of hydrogen peroxide and the time of exposure were the most significant factors for all of the evaluated responses (Figure 2D).

Taking into consideration information provided by Pareto charts, the next only statistically significant conditions where the degradation of atorvastatin (< 10 %) was observed (acid and oxidative degradation) were evaluated.



A) Acid degradation

B) Alkali degradation

C) Thermal degradation

D) Oxidative degradation

Fig. 2. Pareto chart representing the relationship between each factor and their interactions for various conditions of degradation, where the red line is a Bonferroni limitation line.
A: Stressor strength HCl / NaOH or H_2O_2 respectably; B: Temperature; C: Time of exposure
Fig. 2A: Acid degradation; **Fig. 2B:** alkali degradation;
Fig. 2C: Thermal degradation **Fig. 2D:** oxidative degradation

3.2.2. *Evaluation of important factors for acid degradation*

In this work, it was found that the degradation rate of atorvastatin (and the formation of Atorvastatin Impurity H) is strongly dependent on the amount of hydrochloride acid added. As can be seen from the values of the coefficients (b_1 and b_3) for acid hydrolysis, the strength of hydrochloric acid and time of exposure have a positive effect, meaning that the increase in the concentration of hydrochloric acid or prolonged exposure to it, is followed by an increase in degradation.

Values of the coefficients for acid degradation for amounts of total impurity showed that the interaction between time of exposure and temperature has a significant effect on the degradation process, although, individually, these variables were not significant, within the range evaluated, at the 95% confidence interval. This highlights the advantage of the proposed methodology, because this synergistic interaction might be overseen by a traditional approach for conducting degradation studies.

The analysis of the percent of formed Atorvastatin Impurity H during acid degradation follows the same pattern as the Total Impurities discussed above.

In acid degradation, as presented in Table 1, the formation of the major unknown impurity with relative retention time (RRT) 0.74 was not significantly affected and therefore it was not evaluated in this case.

3.2.3. *Evaluation of important factors for oxidative degradation*

Atorvastatin in this study was found to be most affected by oxidative degradation. The obtained results clearly show that in oxidative hydrolysis, the strength of hydrogen peroxide has a positive effect, which means that an increase in the concentration of hydrogen peroxide increases degradation. Also, it has been shown that an increase in the concentration of hydrogen peroxide at optimum temperature increases the rate of degradation continuously. Combination of temperature and strength of hydrogen peroxide has a positive effect, hence a higher strength (30 % H_2O_2) and temperature of 60 °C will give more than 30 % degradation in one hour. This is probably due to the fact that H_2O_2 decomposes into oxygen and water at high temperatures, affecting the pyrrole ring, which consequently results in more extensive degradation. In addition, when the temperature increases, the percentage of degradation also increases (Fig. 2D).

The most susceptible impurities to oxidative degradation were Atorvastatin Impurity H and Impurity with an RRT 0.74. Therefore, besides the percentage of total impurities, the influence of the main factors responsible for their formation was investigated using the proposed full factorial design.

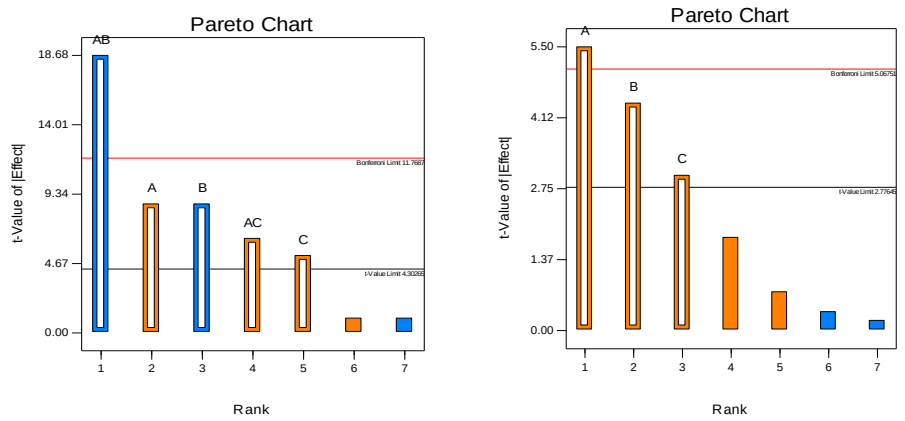
The analysis of variance (ANOVA) of regression parameters for both models shows that they are significant. as presented in Table 6 (F-value of 113.53 and 19.57 and a low probability value Prob > F = 0.0088 and 0.0075, respectably). The correlation coefficient has high values in both models, indicating that only a small percentage of the total variation could not be explained by the empirical model (Table 4).

Furthermore, the representative Pareto charts were plotted as presented in Figures 3a and 3b, for Atorvastatin Impurity H and Impurity, with an RRT 0.74, respectively. From the presented Pareto charts, it was concluded that the most effective parameters in the oxidative degradation for formation of Atorvastatin Impurity H were the concentration of hydrogen peroxide and temperature.

The interaction between the concentration of hydrogen peroxide and temperature was the most influencing interaction. However, the interaction between concentration of hydrogen peroxide and time of exposure was the least influencing parameter. The interaction between concentration of hydrogen peroxide and temperature, as well as their additive effect was shown to have a negative effect on the formation of Impurity H. This means that the degradation falls when the factors passed from the low levels to the high levels.

For correct evaluation of the influence of main factors responsible for formation of the Impurity with RRT 0.74, the obtained values need to be transformed. Therefore, the actual values used in the model were transformed to their squared root values. This transformed value predicted a positive influence of each variable selected for the present study, but the formation of this impurity is mainly affected by the straight of hydrogen peroxide.

These results accentuate another advantages of the proposed design, because performing the experiments in such a manner generates an explanatory model that may be used to predict the behavior of the unknown impurity in a single experiment.



a) Atorvastatin Impurity H **b)** Impurity RRT 0.74

Fig. 3. Pareto chart representing the relationship between each factor and their interaction for oxidative degradation where red line is Bonferroni limitation line. A: Stressor strength H_2O_2; B: Temperature; C: Time of exposure. **Fig. 3a:** Atorvastatin Impunity H; **Fig. 3b:** Impunity RRT 0.74.

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ANOVA for 2^n full factorial design for different type of degradation evaluating the % Atorvastatin Impurity H and % of Unknown Impurity RRT 0.74

	Sum of squares	df	Mean square	F-value	P-value Prob > F	
Atorvastatin Impurity H						
Model	2.56	5	0.51	113.53	0.0088	significant
A – Strength H_2O_2	0.34	1	0.34	75.42	0.0130	
B – Temperature	0.34	1	0.34	75.42	0.0130	
C – Time	0.12	1	0.12	27.15	0.0349	
AB – Strength H_2O_2**Temperature*	1.58	1	1.58	349.10	0.0029	
AC – *Strength* H_2O_2**Time*	0.18	1	0.18	40.56	0.0238	
Residual	9.025E-003	2	4.531E-003			
Cor Total	2.57	7				
Impurity RRT 0.74						
Model	8.67	3	2.89	19.57	0.0075	significant
A – StrengthH_2O_2	4.46	1	4.46	30.24	0.0053	
B – Temperature	2.87	1	2.87	19.43	0.0116	
C – Time	1.34	1	1.34	9.06	0.0396	
Residual	0.59	4	0.15			
Cor Total	9.26	7				

Aberrations:
df: degree of freedom. Cor Total: corrected total sum of squares. Values of Prob > F: less than 0.05 indicates models are statistically significant

Next, in the evaluation phase, response surface plots were generated for the most significant factors for each of the various degradation conditions, providing information about the degradation in regions. Each response surface plot represents a number of combinations of two test variables with all other variables at low levels.

The response surface plot for acid degradation was generated by keeping the time of exposure at the maximum value (Fig. 4 a). As discussed before, an increase in the concentration of HCl and longer exposure is followed by an increase of the amount of total impurities.

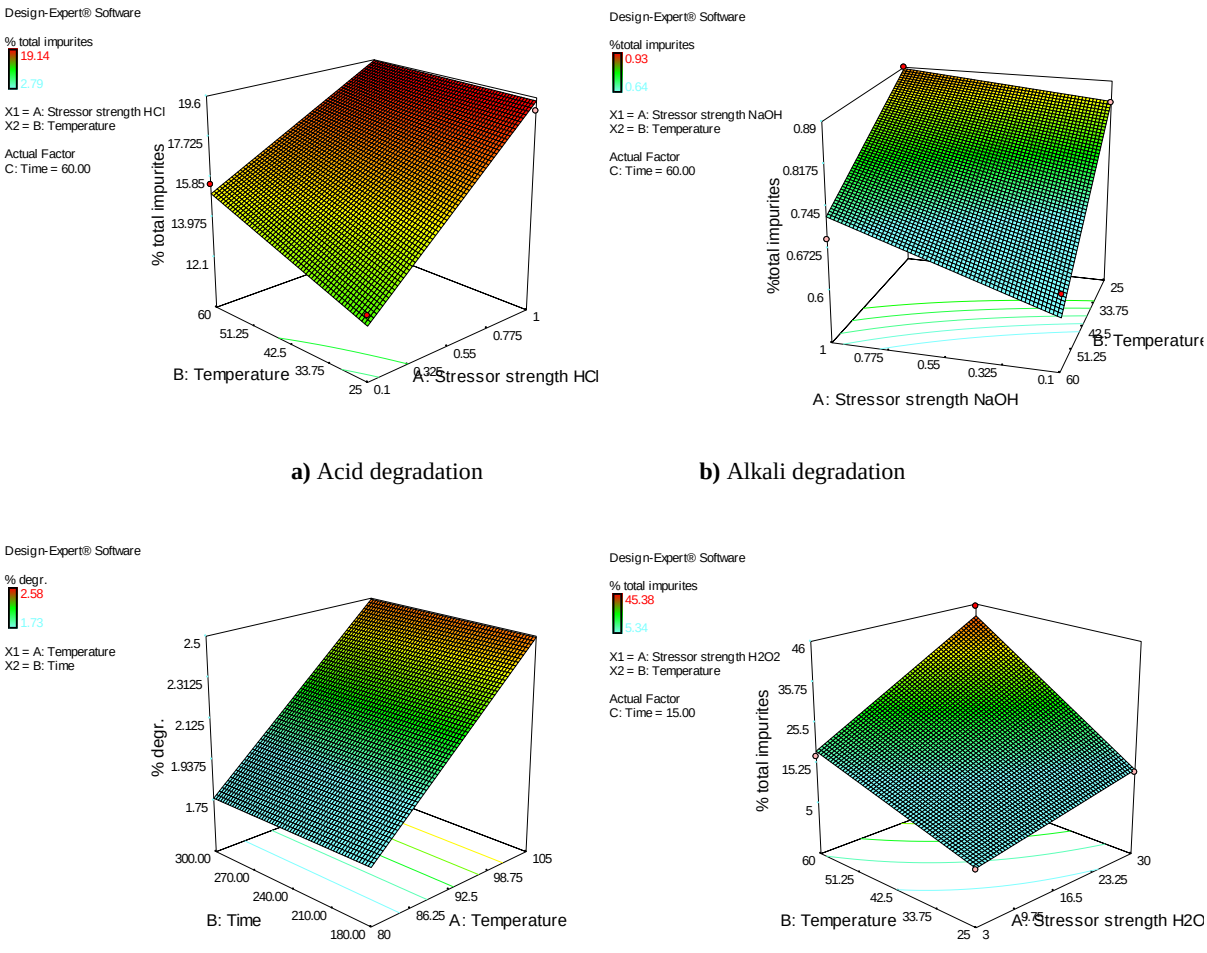
In alkali degradation response, the surface gradually increased with an increase in concentration from 0.1 M to 1 M NaOH. However, as discussed above, this model could not be used for prediction.

The response surface plot for oxidative degradation was generated by keeping the temperature at a minimum value (Fig. 4d). Additionally, when evaluating the obtained results, it can be seen that atorvastatin is very sensitive in oxidative condi-

tions; in some of the experiments, the degradation of nearly 50 % was observed. Performing the experiments using DoE allows an overview of the degradation behavior in a wider region, so the risk of obtaining irrelevant results from secondary degradation is minimized, thus pointing out the additional advantage of the proposed design. A response surface plot offers a good, information-rich presentation of the experimental results. Raw experimental data would be meaningless without models able to provide meaning from observable fact.

The response surface plot obtained from thermal degradation study demonstrated that an increase in temperature from 80 °C to 105 °C favored the degradation significantly. This plot demonstrated that the combination of temperature and time of exposure has a positive effect meaning that longer exposure at a temperature of 105 °C will result in higher degradation, but the maximum obtained degradation was about 3 %. For thermal degradation, it has been observed that 3 % degradation would be achieved by heating the solution at 105 °C for 5 h. When these conditions were adopted in practice, the resulting degradation was 2.68 %.

Photolytic studies were performed in a classical manner and a degradation of about 1.76 % has been obtained after exposure to UV light for 2 days.



a) Acid degradation

b) Alkali degradation

c) Thermal degradation

d) Oxidative degradation

Fig. 4. 3D response surface plots showing the desired degradation under various conditions.
Color change from blue to red represents increasing desirability (min max)
Fig. 4a: Acid degradation; **Fig. 4b:** Alkali degradation; **Fig. 4c:** Thermal degradation**; Fig. 4d:** Oxidative degradation.

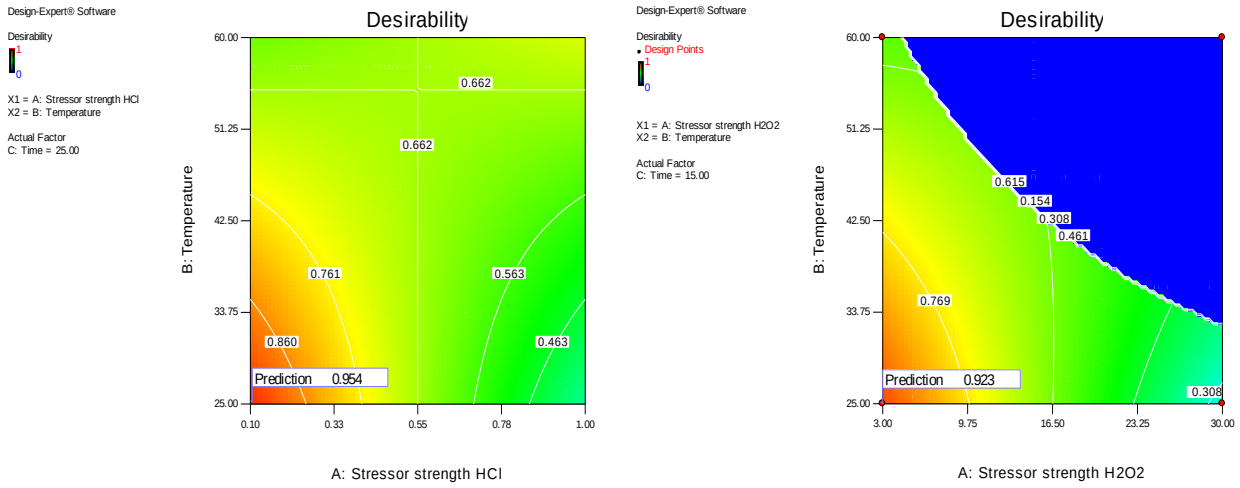
3.2.4. *Selection of optimal conditions*

In this study optimal conditions for acid degradation were chosen using a desirability function, where the % of Impurity H was targeted to minimal value and targeted degradation was set in the range from 5–20 % following the general literature recommendations [5, 12, 13]. No specific limitations were imposed to the % of Impurity RRT of 0.75, as its value falls within the specification limits (<0.1%) in all 8 experiments. Optimal conditions for oxidative degradation were chosen using a desirability function, where % of Impurity H was targeted to a minimal value and targeted degradation was set in the range from 5–20 % following the general literature recommendations [3, 5]. In this case, limitations for % of Impurity RRT 0.75 were set to 0.1 %.

From the desirability plot presented in Figure 5, it can be concluded that the set of coordinates producing high desirability value (D HCL = 0.954 and D H_2O_2 = 0.923) were 0.1 M HCl, 25 °C and 25 minutes (Fig. 5A) and 3 % H_2O_2, 25 °C and 15 minutes (Fig. 5B), respectively.

These conditions were selected as experimental conditions in the verification study. The predicted response values corresponding to the above optimum conditions are given in Table 7. Comparison between the obtained and predicted results was made and no noticeable difference was clearly observed (Table 7). The results of the experiments confirmed that the chosen model was adequate for reflecting the expected optimization. Good predictability of the desirability plot provides valuable information about proposed methodology, saving considerable amounts of chemicals and experimental time.

Representative overlaid chromatograms for all degradation conditions obtained with the confirmatory experiments are presented in Figure 6.



A) Acid degradation B) Oxidative degradation

Fig. 5. Optimization of the selected responses by means of the desirability function. The red area corresponds to the optimum conditions while time maintained constant A) acid degradation; B) oxidative degradation

T a b l e 7

Comparison of experimental and predictive values of different responses under optimal conditions

Parameters	Predicted (%)		Obtained (%)		Predicted error	
	Total imp.	RRt 0.75	Total imp.	RRt 0.75	Total imp.	RRt 0.75
Acid degradation	5.00	0.05	5.03	0.056	0.60	–10.71
Oxidative degradation	5.75	0.77	5.34	0.73	–7.13	5.48

Predicted error = (Obtained values – Predicted)/Predicted*100) BDL- Below disregard limit (0.05%)

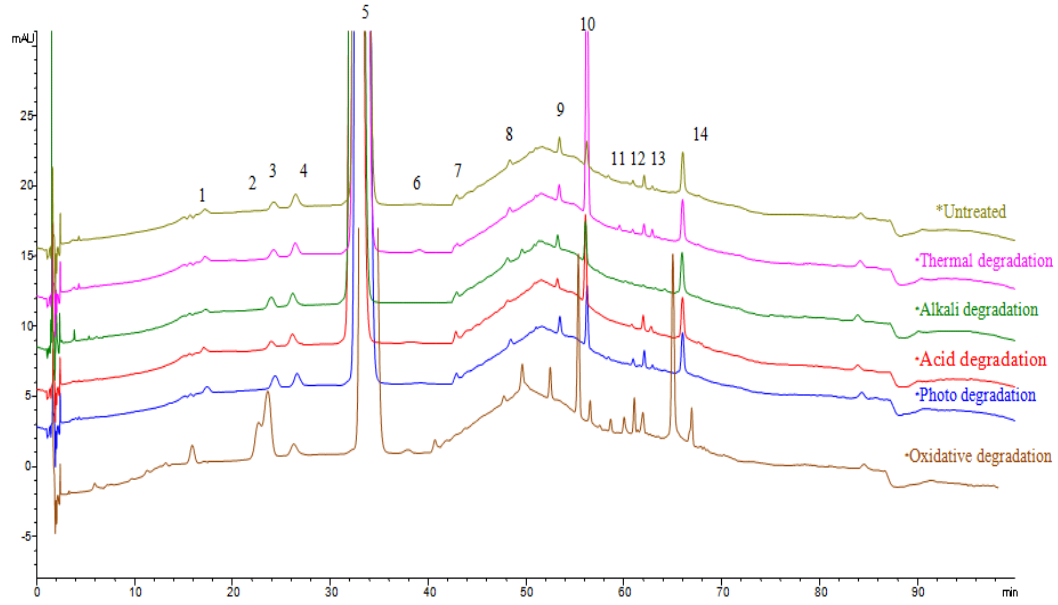


Fig. 6. Overlaid chromatographs from all degradation conditions where 1 – Atorvastatin Impurity F; 3 – RRT 0.74; 4 – Atorvastatin Impurity A; 5 – Atorvastatin; 7 – Atorvastatin Impurity C; 9 – Atorvastatin Impurity G; 10 – Atorvastatin Impurity H; 13 – Atorvastatin Impurity D1; 14 – Atorvastatin Impurity D2; 2,6,8,11,12 – unknown impurities

To carry out deeper analysis of the behavior of atorvastatin, mass spectra and fragmentation patterns of all impurities were recorded, analyzed with Mass Frontier 7.0 fragmentation software and confirmed by data published in the literature.

Along with the known impurities, this approach enabled the identification of additional impurities revealed by the optimized stress conditions, which might be overseen by a traditional approach for conducting the degradation studies. However, all of the additional impurities were under the disregard limit (0.05 %) and did not affect the result. This could be the subject of other studies discussed elsewhere. The obtained results also indicate that the two used methods (proposed LC/MS and compendia) yield very similar results for evaluation of the degradation process, and confirm the suitability of the proposed methodology.

The use of an experimental design to identify the theoretical values of variables for optimum degradation was successful, because, when the proposed parameters were put in practice, the obtained results matched the predicted degradation.

In fact, the most significant advantage of the present methodology is the simplicity of the sample preparation, since measurements are made directly on the liquid samples and optimum degradation was achieved in minimal experimental trials.

The implementation of experimental design in the optimization of experimental conditions in a forced degradation study gives better data quality with less laboratory work, and leads to a decrease in a cost of analysis.

4. CONCLUSION

The proposed methodology represents an efficient and easily accomplishable approach for searching for optimum degradation conditions.

This study showed that a full factorial experimental design is an excellent tool and could be successfully used to develop empirical equation for the prediction and understanding the degradation process. The obtained results showed sufficiently good correlation between the experimental data and predictive value throughout the studied parameters. This suggests that proposed full factorial design approach can replace the trial and error approach used to achieve optimum degradation in forced degradation studies.

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