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Quality by Design Approach for the Development of UHPLC Method indicating the Stability of Acyclovir in Gamma Radio-Sterilized Ophthalmic Polymeric Hydrogel Formulation

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Abstract

The stability of Acyclovir in a new polymeric hydrogel ophthalmic formulation towards gamma radio-sterilization was studied using UHPLC method. The gamma radio-sterilization of the formulation may result in degradation of the main active material (Acyclovir). So, the free radicle scavenger Pyridoxine was added to protect the drug from degradation during sterilization. Quality by Design strategy was followed to achieve the challenging separation between Acyclovir and Pyridoxine and other formulation excipients using UHPLC. Acetonitrile containing 0.5% acetic acid: 0.5% acetic acid (70:30) and 0.86 mL/min flow rate was found to give the best separation results. Acyclovir and Pyridoxine were detected and quantified using PDA detector at 254nm and 292nm, respectively. The results showed that Pyridoxine protected Acyclovir from complete degradation during gamma sterilization where the recovery was found to be 50.12%. The developed method was validated as per ICH guidelines and statistically compared with detailed method with some modifications. Separation between Acyclovir and Pyridoxine and other formulation excipients using UHPLC.

Keywords: Stability; UHPLC; Acyclovir; Ophthalmic; Radio-Sterilized

1. Introduction

Increasingly individuals around the world are influenced by extreme eye disease, without satisfactory treatment, these diseases will in the long run lead to serious visual problems or blindness. Unfortunately, most currently available therapeutic approaches have major failings, Eye drops usually suffer from low bioavailability because of fast nasolacrimal drainage (1).

An effective therapeutic level is a big challenge because of low patient compliance result from short interval doses and high drug concentration (2). Additionally, absorption of the drug drained through the nasolacrimal canal may cause systemic side effects. To decrease systemic side effects various vehicles, such as suspensions, ointments and inserts, have been created (3).

Indeed, in spite of the fact that a few enhancements over conventional liquid dosage forms have been developed, these drug delivery systems have many problems such as blurred vision and foreign body sensations. Regarding patient compliance, eye drops that stay in contact with the cornea and sustained drug release over extended periods of time would be perfect.

Hydrogels may offer several advantages over the other dosage forms that have been created for eye preparations, because of their favorable transparency and mechanical flexibility and compatibility with active pharmaceutical ingredients (4). Numerous distinctive polymers, either of natural or of synthetic origin, have been examined for their safety in ophthalmic delivery systems (5). Hyaluronic acid (HA) is one of the most utilized polymers is, a naturally occurring polysaccharide. Pure HA, cannot provide the mechanical properties necessary for most medical applications (6).

In the present study, the hydrogel was developed by cross-linking HA and polyvinylpyrrolidone (PVP) hybrid for constructing ophthalmic drug delivery system under aqueous conditions.

Compared to chemically cross-linked hydrogels, which require extensive hydrogel purification to get non-toxic cross-linking agents made of tiny molecules, materials for biological use, physically cross-linked hydrogels (radiation-polymerization) are advantageous because no possible toxicity has been discussed(6).

A plethora of methods have been adopted to promote crosslinking including chemical and physical methods.

Ionizing radiation has been used as a novel facility to initiate polymerization and/or crosslinking reactions, without the need to use carcinogenic materials (initiators and/or cross-linkers), due to the toxicity of chemical crosslinking agents such as glutaraldehyde and H2O2 (7). Ionizing radiation has been used as a novel facility to initiate polymerization and/or crosslinking reactions, without the need to use carcinogenic materials (initiators and/or cross-linkers), due to the toxicity of chemical crosslinking agents such as glutaraldehyde and H2O2 (8). Due to these benefits, the method of choice for creating hydrogels for application in biomedicine is radiation-induced polymerization and crosslinking of copolymers (9).

Gamma radiation has been used in the past to create across-linked structures (10).

Water soluble polymers have been shown to be capable of intramolecular cross-linking in diluted solutions (polymer concentration must be below enough to avoid intermolecular cross-linking) (11).

As a high energy source, gamma rays are used in the process to create free radicals in the polymer. Şen and Avc have presented a productive approach of hydrogel assembly based on high-energy radiation. These hydrogels were made by irradiating aqueous poly (N-vinyl-2-pyrrolidone) (PVP) solutions. When PVP and hydroxyl radicals produced during water radiolysis interact, macro radicals are created (9).

Therefore, the use of radiation could be regarded as a manageable way for creating drug delivery systems(8).

Formulations for ophthalmic preparation should be essentially sterilized. To ensure an extended shelf life for the product, the manufacturing process must be carried out in sterile conditions or should include terminal sterilization (7). There are several sterilization methods that could be used for ophthalmic formulations in which ingredients subjected to sterilization separately and then combined in a superior environment that is intended to reduce contamination but not ensure sterility (12). These include autoclaving, filtration, gaseous ethylene oxide, and the use of formaldehyde(13).

Gamma sterilization could be used in terminal sterilization of sealed packaged products. The DNA or RNA of bacteria, fungi, yeasts, and viruses is harmed by gamma sterilization (14). One of the approaches that exist to reduce the effects of gamma irradiation is to use gamma radiation-resistant excipients, including free radical scavengers in the formulation (13).

It is safe to recommend radio-sterilization using ionizing radiation for the terminal sterilization of solid pharmaceuticals (15, 16). Nevertheless, high degradation is reported after irradiation of drugs in aqueous solutions (17). However, drug degradation could be avoided by the addition of reactive species scavenger. That, could protect the drug from degradation (18).

In this work, since pyridoxine (vitamin B6) is a tiny molecule with several chemical bonds, it was chosen as the radioprotective excipient for the antiviral medicine acyclovir, which is used as a model drug for ocular preparation.

Many methods were found in the literature for Acyclovir determination using HPLC either in dosage forms or human plasma (19-22). Literature survey revealed that all the developed HPLC methods have been achieved using one factor at a time (OFAT) strategy .This needs too many experiments for method development. Also, OFAT strategy does not take into consideration the main and interaction effects of the studied variables.

The study developed a chromatographic method for separation of the very challenging mixture Acyclovir and Pyridoxine in the same formulation of PVP hydrogel. For this reason, Quality by Design (QbD) strategy was exploited to overcome the aforementioned disadvantages and also to provide complete comprehension of how the important factors interrelate to influence resolution effectiveness and overall retention times of the analytes (Acyclovir and Pyridoxine). QbD was firstly introduced by FDA cGMP for the twenty-first century (23) and the international conference on Harmonization (ICH) guidelines Q8(R2) (24).

The first step in QbD is the preliminary trials, which provide the analyst with the general understanding of the required conditions of analysis. The second step is screening, which determines the potential significant variables which could affect the efficiency of separation (25). The final step is optimization, which gives a full and in-depth understanding of the critical parameters that affects the separation and enables the analyst to figure out the most appropriate conditions

required for the top efficient separation (25). QbD aids in obtaining the desired response and optimizes the variables that give the maximum, minimum, or target response.

The aim of the present study was to exploit QbD approach to develop a multivariate UHPLC model for predicting and optimizing the different variables to study the stability of Acyclovir in Gamma Radio-Sterilized Ophthalmic Drug Polymeric Hydrogel Formulation.

2. Materials and methods

2.1. Materials

Acyclovir and pyridoxine (purity > 99%) were acquired from Sigma–Aldrich (St. Louis, MO, USA) and stored in the dark at room temperature. Hyaluronic Acid (HA) (95.7% assay, M w: 1.48×106) was obtained from Pacific Co (Seoul, Korea). PVP (M w: 360,000) was purchased from Sigma Chemical Co. (USA). Acetonitrile (HPLC grade; Fisher Scientific, UK). Deionized water was generated in our laboratory with a Milli-Q system from Millipore (Bedford, MA, USA). Nitrogen (N28 purity) was supplied by Air-Liquide (Li`ege, Belgium). All other chemicals were extra pure reagent grade and were used as received. Chemical structures of Acyclovir and Pyridoxine are presented in electronic supplementary material.

2.2 Instrument and Software

Chromatographic system Schimadzu LC-2040C 3D PLUS nexera-i and triple quadrupole MS 8040(Japan) equipped with PDA detector (LC-2030/2040 PDA), LC-2040 pump and 4-line degasser were used to achieve the separation. The Chromatographic system Schimadzu LC-2040C 3D PLUS nexera-i and triple quadrupole MS 8040 were used to achieve the separation.

2.3. Methodology

2.3.1 Preparation of standard stock solutions

Separately, standard stock solutions of pyridoxine and acyclovir were created in 100-mL volumetric flasks by combining 50 mg of the standard powder with acetonitrile and deionized water (50:50).The analytes' standard stock solutions were then diluted with the mobile phase to produce standard working solutions. (100 µg/mL).

2.3.2 Preparation of Hydrogels

The stated methods were used to prepare different HA-PVP hydrogel compositions. PVP K90 and HA were dissolved in 50 mL of tri-distilled water at a weight-percentage ratio of 1-10. (Spinks and Woods, 1990). Medicated hydrogel samples were prepared by adding Acyclovir at concentration 5 mg/mL (which are in line with the concentration of the commercial products) either without excipients (0 or with 10 mg/mL pyridoxine hydrochloride. These combinations of solution were placed in glass vials, shielded from light, filled with nitrogen, sealed, and either not exposed to radiation () or exposed to radiation at room temperature. produced from ^{60}Co source (national center for radiation research and technology (NCRRT), Egypt). Using Gamma cell at 10, 20, 25, 30 35 and 50 kGy dose (, respectively) (dose rate: 12.5 kGy h^{-1}) with and without pyridoxine hydrochloride. In order to examine how the absorbed dose affects the drug's breakdown as well as how pyridoxine affects the drug's protection.

2.3.3 Preparation of the analytes' solution from the polymeric hydrogel formulation

A 250 mL beaker was filled with 200 milligrams after being weighed. Then, 100 mL of a 1:1 mixture of deionized water and acetonitrile were added, agitated for 15 minutes with a magnetic stirrer, and sonicated for 15 minutes. Afterward, dilute with the mobile phase to 200 mL. To prepare 10 mL for examination, a syringe filter measuring 0.2µm was placed within the injection vial. The appropriate computed regression equations were used to calculate the concentration of the analytes in the prepared samples.

2.3.4 Chromatographic conditions

Separation of the analytes in the radio-sterilized polymeric hydrogel formulation was carried out on Shim-pack GISS C-18, (50 x 3mm, 3µ) Column. The injection volume was 10 µL. The produced optimization model suggests the following conditions for obtaining the best efficient separation and quantification for the analytes to indicate acyclovir stability: Acetonitrile containing 0.5% acetic acid: 0.5% acetic acid (70:30) and 0.86 mL/min flow rate was found to give the best

separation results. Acyclovir and Pyridoxine were detected and quantified using PDA detector at $\lambda_{254\ nm}$ and $\lambda_{292\ nm}$, respectively.

2.3.5 MS Spectrometry conditions

The triple quadrupole MS 8040 with an ESI interface operating at 4.5 kV was linked to the LC. DL and Heat block temperatures of 2500C and 4000C, respectively, were used with the positive ionization mode. Nitrogen gas flow rates for nebulizing and drying were 3L/min and 15L/min, respectively. The entire mass range, from 100 to 1000 m/z, was covered by the scan.

2.3.6 Validation

2.3.6.1 *Linearity and range*

Acyclovir was placed in precisely measured aliquots from their standard working solutions into several 10-mL volumetric flasks, and the volume was then brought to 10 mL with mobile phase. The six concentrations in the calibration standards span the full range of acyclovir concentrations, from 1 to 100 µg/mL. Using the appropriate chromatographic settings and a flow rate of 0.86 mL/min, the samples were each independently injected into the column. The average peak area of Acyclovir was displayed against the matching concentrations after each sample undergone three analyses. After that, regression equations were calculated.

2.3.6.2 *Accuracy*

The proposed method was used to determine three replicates at various Acyclovir concentrations in order to verify the accuracy of the results. The concentrations were derived from the respective regression equations, and the Percentages recoveries from these indicated excellent precision of the suggested procedure.

2.3.6.3 *Precision Repeatability*

Under the same chromatographic conditions, three injections of each of the three Acyclovir concentrations were made intra-daily. There was a calculation of the relative standard deviations.

2.3.6.4 *Intermediate precision*

For the examination of the three selected concentrations, the abovementioned process was done three times daily on different days. There was a calculation of the relative standard deviations.

.2.3.6.5 *Limit of quantitation (LOQ) and limit of detection (LOD)*

The minimal concentration at which the analyte may be reliably identified is established by comparing measured signals from samples with known low amounts of analyte with those of blank samples. For calculating the detection limit, a signal-to-noise ratio of 3:1 is often considered acceptable, while for estimating the quantitation limit, a ratio of 10:1 is employed.

3. Results and Discussion

Introduction of polymers to the formulation, which allowed for longer contact between the active ingredient and the corneal surface, was one of the first changes made to conventional forms of ophthalmic drugs. This increased the active ingredient's bioavailability by lengthening its contact with the cornea. Drug distribution can be accomplished using cross-linked polymeric substances (hydrogels). They are preferred for these uses because they are nontoxic and have a three-dimensional structure that can regulate medication release (6). In this study, Hyaluronic acid (HA) and polyvinylpyrrolidone (PVP) hybrid was used to prepare ophthalmic hydrogels using gamma radiation as physical cross-linking tool for preparing safe and smart drug delivery system under aqueous conditions. After HA/PVP/water blend was irradiated at gamma radiation dose of 25 kGy (required for sterilization, Typically, terminal sterility can be attained with 25 kGy and a sterility assurance level (SAL) of 10^{-6} (26); Due to the presence of two reactive groups at the C-2 and C-6 locations in saccharide groups, a chemical interaction between polysaccharide (Hyaluronic acid (HA)) and synthetic polymer molecules would result (27). Moreover, vinyl groups in PVP and carboxylic groups in HA could be initiated by hydroxyl radicals. A cross-linked PVP branching chains networks is now created by the combining the two nearby polymeric radicals (6). In contrast, if the radiation's energy is high, the opposite process of crosslinking(degradation)occurs, in which C-C bonds are ruptured (6).

According to FDA, ophthalmic preparations are sterile preparations intended for application to the eye. Aseptic processing and terminal sterilization are two different ways to make sterile pharmacological products, and these differences are fundamental. Irradiation is frequently used for terminal sterilization to sterilize the final container. In contrast, an aseptic method involves sterilizing the medication product, container, and closure separately before combining them in a very high-quality environment that is meant to reduce contamination but not to ensure sterility (25). But in radio sterilization, irradiation aqueous solutions produce reactive species by radiolyzing water (primarily •OH, •H, and eaq), which then react with the solute (indirect action), giving birth to radiolytic products. Only a few investigations have been done on the radio-sterilization of aqueous solutions (15).

Aqueous solutions could be radio sterilized under ideal irradiation conditions by adding radio-protective excipients and lowering the rate of medication breakdown (the final purpose being the terminal sterilization). Pyridoxine (vitamin B6) was selected as a radioprotective excipient, as it reacts with both the hydroxyl radical and the aqueous electron (17). Consequently, pyridoxine is expected to strongly reduce the loss in Acyclovir due to gamma irradiation by scavenging reactive species (17).

The UHPLC-PDA technique was utilized to indicate the stability of Acyclovir after irradiation with different doses of gamma radiation, in presence and absence of the free radicle scavenger Pyridoxine.

Liquid chromatography is the most widely used analytical technique for separation and quantification of analytes in both industry and academia. OFAT methods were used which require large number of experiments and in addition, with this strategy, it is impossible to fully comprehend how important factors interact to influence the separation and retention parameters. So, the challenge was to use a Quality by Design strategy to develop UHPLC method for Acyclovir stability towards sterilization using different dose of gamma radiation in presence and absence of the free radicle scavenger Pyridoxine.

Response surface study with central composite design was applied for optimizing the studied factors. The produced random 16 runs for the screening step and the other 13 runs for the optimization step are presented in the Electronic Supplementary Material (Table S1 and S2).

Preliminary trials

Several preliminary trial runs were tried for general understanding of the factors that have the greatest effect on resolution and run time. It was found that a mobile phase composed of acetonitrile and buffer (pH=3) or buffer (pH=7) resulted in partial or complete overlapping of both peaks of Acyclovir and Pyridoxine even if we changed the mobile phase proportions. Then, when acetonitrile and 0.1% acetic acid (20:80 v/v) were used as the mobile phase, excellent resolution could be achieved in less than two minutes. So, the ratios of acetonitrile and acetic acid in the mobile phase, the flow rate, the strength of acetic acid solution, and the column temperature were studied as critical factors that could affect the resolution and run time.

Screening Step

Regular Two-Level Factorial Design was adopted for the screening of all the critical factors and how they could affect the studied responses: Resolution between the two analytes (R) (Acyclovir and Pyridoxine), Number of theoretical plates for Pyridoxine (N_1), Number of theoretical plates for Acyclovir (N_2), Run time (Rt). Two levels for each factor were used: % Acetonitrile at 20% and 60%, % Acetic acid at 0.5% and 1.5%, Flow rate at 1 mL/min and 2 mL/min, and Oven temperature at 30^0 and 45^0 C. The total number of runs for the screening step was (2^4) = 16, as presented in the Electronic Supplementary Material (Table S1). The factors were studied by Factorial study type using 2 level factorial design type. The process order of the developed screening model was main effects only and the type was factorial model. Alpha = 0.05 was chosen as the level of significance to evaluate the impact of the model terms. The ANOVA for the selected factorial model showed that only two factors are significant. The two factors are % acetonitrile (factor A) and flow rate (factor C). The remaining two factors (% acetic acid and oven temperature) were statistically insignificant factors as presented in Table 1. Figure 1 shows the Pareto charts which indicate the significant factors for each response.

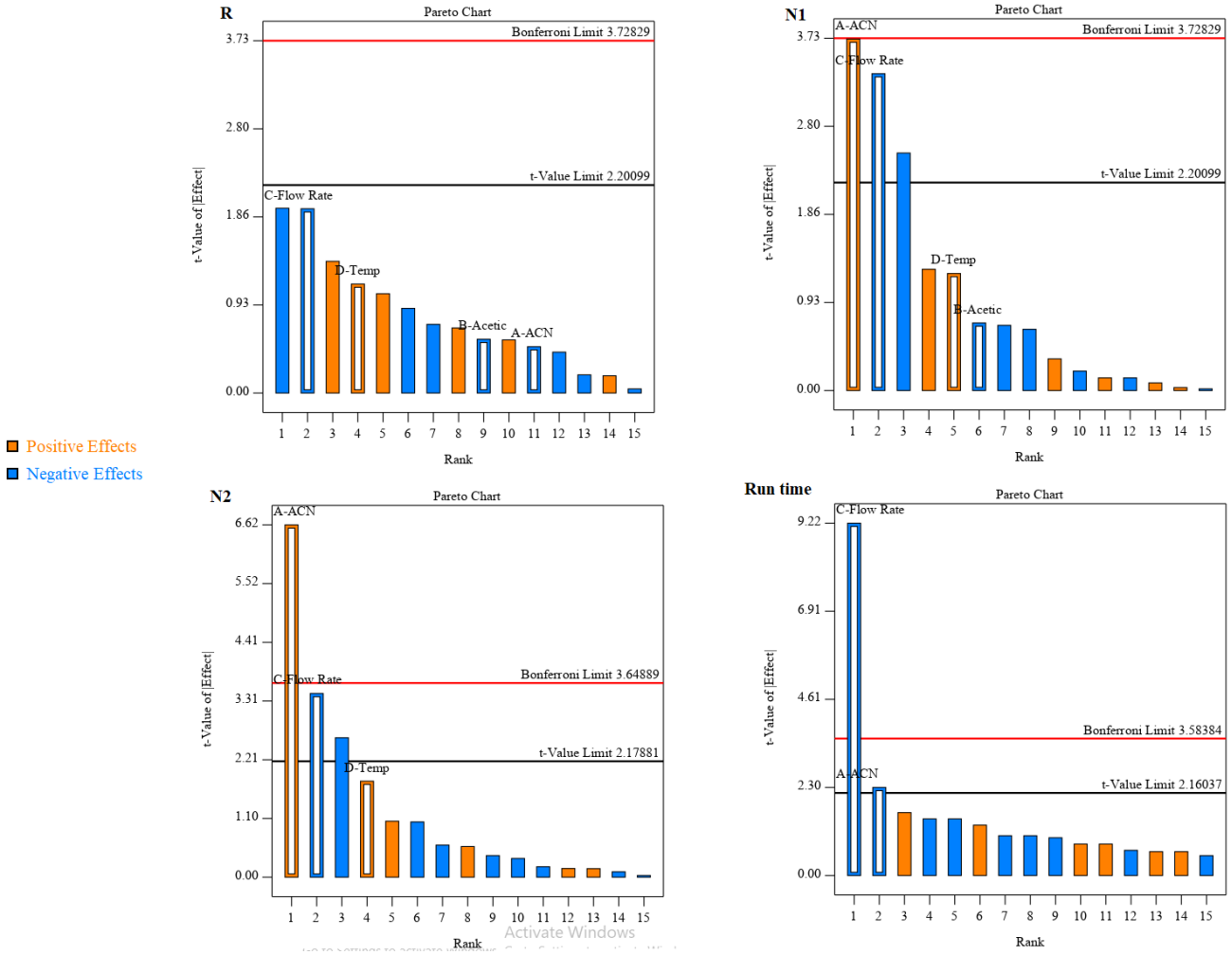

Figure 1: the Pareto charts which indicate the significant factors for each response

Table 1: Figure 1 shows the Pareto charts which indicate the significant factors for each response.

Table 1: ANOVA Results of the fractional factorial design (insignificant interaction effects were excluded)

Item	R		N1		N2		Run Time	
	F	p value	F	p value	F	p value	F	p value
ACN%	____	____	**13.83**	**<0.05**	**43.83**	**<0.05**	**5.31**	**<0.05**
Acetic %	____	____	____	____	____	____	____	____
Flow Rate	____	____	**11.24**	**<0.05**	**11.91**	**<0.05**	**84.93**	**<0.05**
Temp.	____	____	____	____	____	____	____	____
Adjusted R^2	**0.9142**		**0.8073**		**0.8311**		**0.8547**	

Bold values indicate that the independent factor has a significant effect on the selected response

Optimization Step

Here, the critical factors from the screening step were further studied for optimization. Response surface was used as the study type where central composite design was used as the design type. The process order of the developed optimization model was quadratic, and the model type was polynomial. The total number of runs for the optimization step was 13, as presented in the Electronic Supplementary Material (Table S2). The significance level to assess the effect of the model terms was alpha = 0.05. Each factor was studied at 5 different levels to find the optimum conditions for the desired response. Table 2 shows the results of the ANOVA statistical test. Results showed that quadratic model is the suggested

one for three factors (Resolution, N_1, and Run time) where two factor interaction (2FI) is suggested for one factor only which is N_2. The following equations represent the relation between the different responses (y) and the significant factors:

$$Y_{Rs} = a + b1\,(x_1) - b2\,(x_2) + b3\,(x_1)\,(x_2) + b4\,x_1^2 - b5\,x_2^2$$

Figure 2 shows the effect of the studied factors on the corresponding responses in terms of Perturbation charts. The charts show positive effect of % acetonitrile and negative one of flow rate on Resolution, N_1, and N_2. Also, they demonstrate that flow rate has a negative impact on runtime and that acetonitrile % has no bearing on it. Table 2 shows the interaction effects of the various components as a result of the quadratic model.

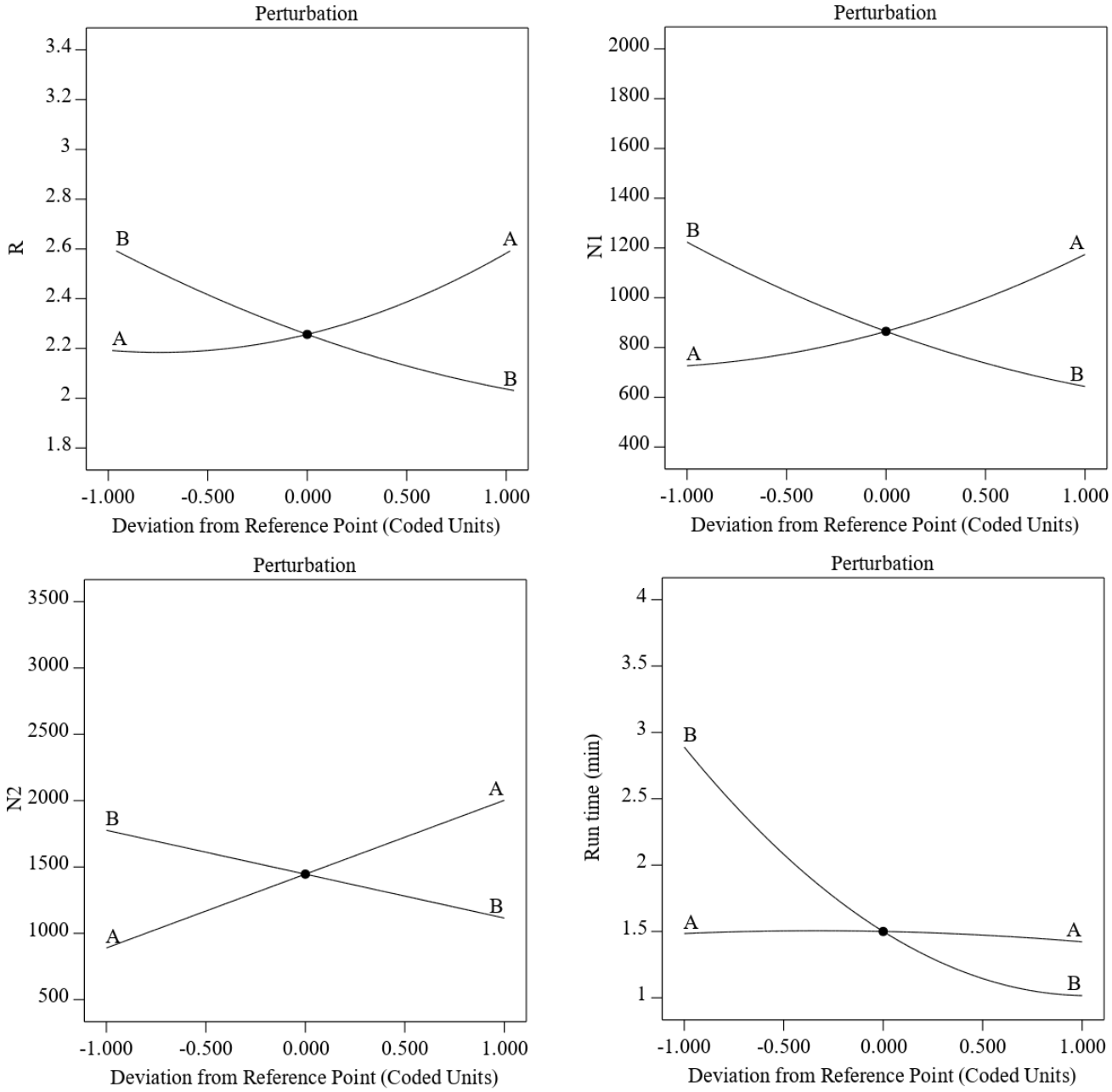

Figure 2: the effect of the studied factors on the corresponding responses in terms of Perturbation charts.

Table 2: shows the interaction effects of the various components as a result of the quadratic model.

Item	R		N1		N2		Run Time	
	coefficient	p-value	coefficient	p-value	coefficient	p-value	coefficient	p-value
Intercept	2.363		432.855		-1880.276		5.461	
A-Aceto	-0.0093	0.0005	12.4983	0.0001	88.072	< 0.0001	0.2717	0.6183
B-Flow Rate	0.154	< 0.0001	172.596	< 0.0001	1190.809	0.0034	244.03	< 0.0001
AB	0.0226	0.0016	-23.4871	0.0009	-37.769	0.0128	0.5434	0.485
A^2	0.0006	0.0059	0.3791	0.0311			0.5316	0.4896
B^2	0.157	0.0898	162.2781	0.0675			49.67	0.0002
Model	Quadratic	0.0001	Quadratic	< 0.0001	2FI	0.0001	Quadratic	< 0.0001

Full understanding of the effects of factors on the responses was attained by both the surface plot and the contour plot as shown in Figure 3 a, b.

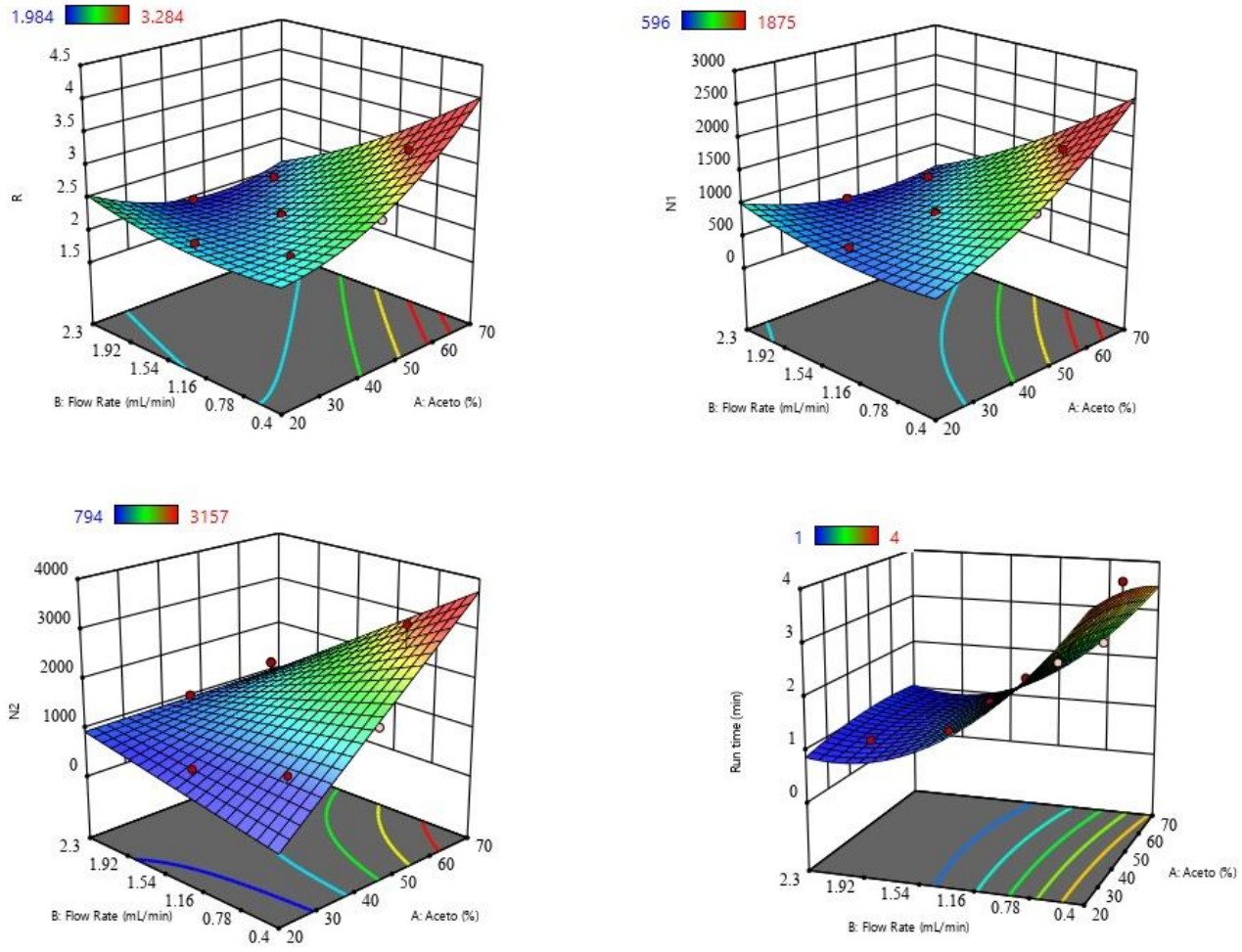

Figure 3 a: effects of factors on the responses was attained the surface plot

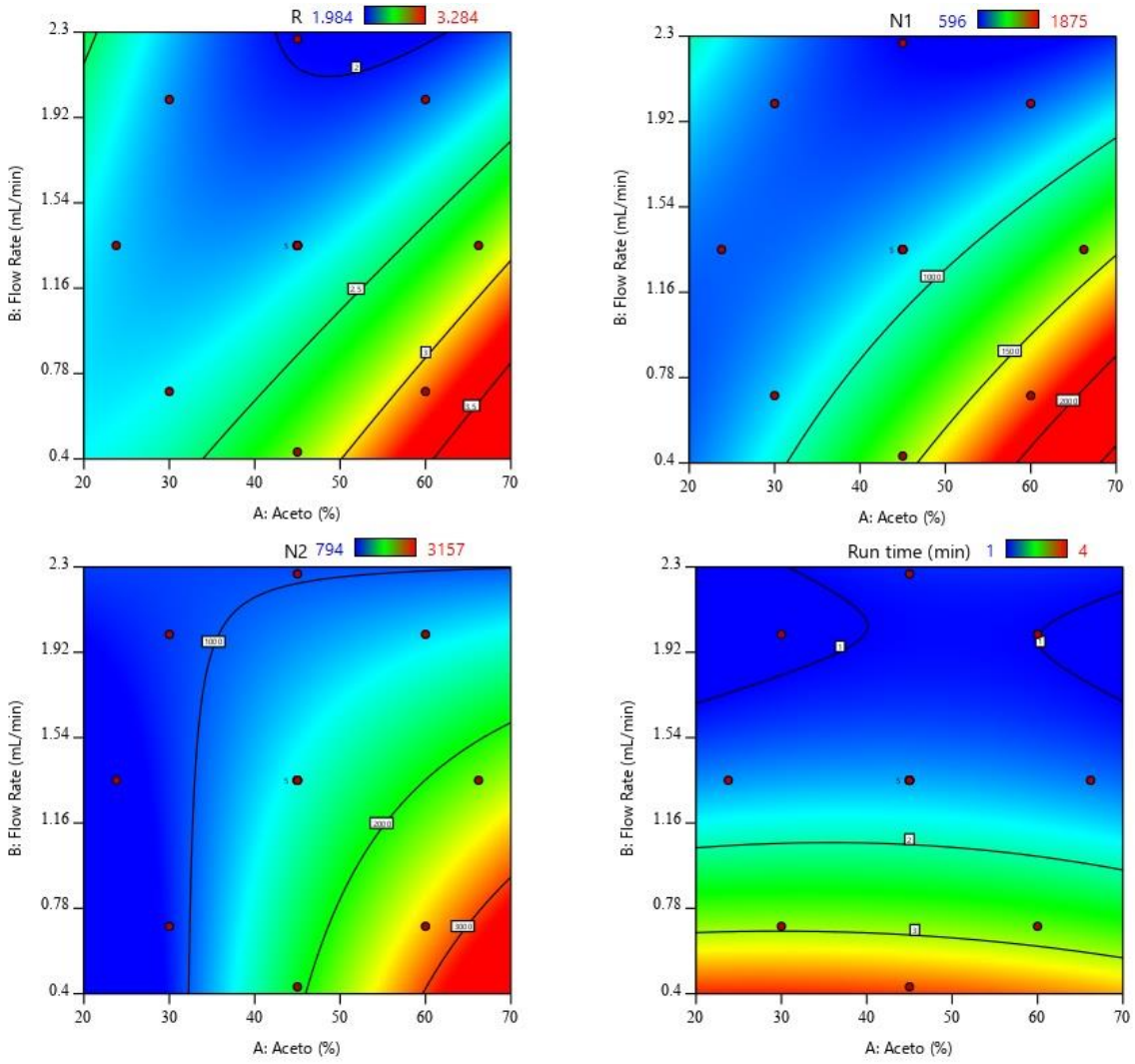

Figure 3 b: effects of factors on the responses was attained by the contour plot

To optimize the responses, the Numerical optimization tool of Design-Expert 11 software was utilized. Resolution, N1, and N2 had their desirability criterion set to maximum, whilst Run time had theirs set to minimum. The optimization tool suggested that 70% acetonitrile and 0.86 mL/min flow rate will be optimum for the optimum performance with desirability of 0.86.

For graphical optimization, the setting was as follows: lower limit of R and N was 2 and 2000, respectively. Where run time was set to 2 minutes. The overlay plot showed the optimum conditions as shown in Figure 4. The areas that fit with the optimization criteria are yellow in color where the areas that do not fit are colored gray. The chromatogram for separation of Acyclovir and Pyridoxine is shown in Figure 5.

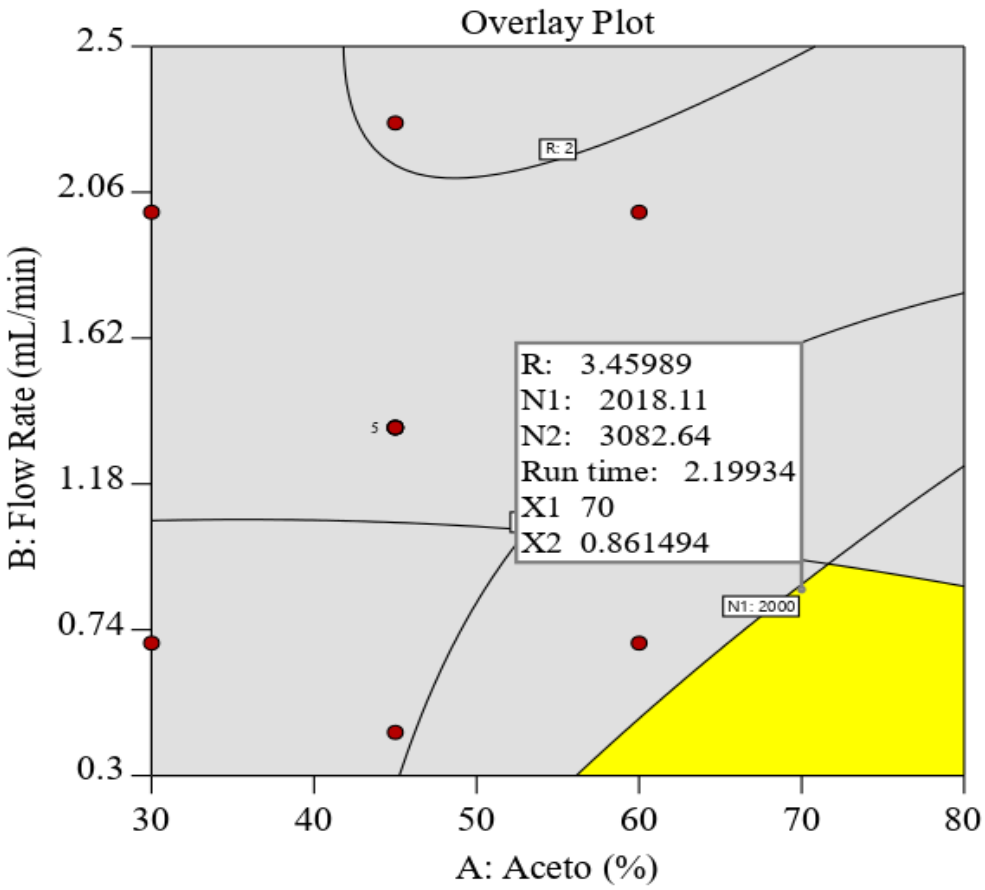

Figure 4: The areas that fit with the optimization criteria are yellow in color where the areas that do not fit are colored gray.

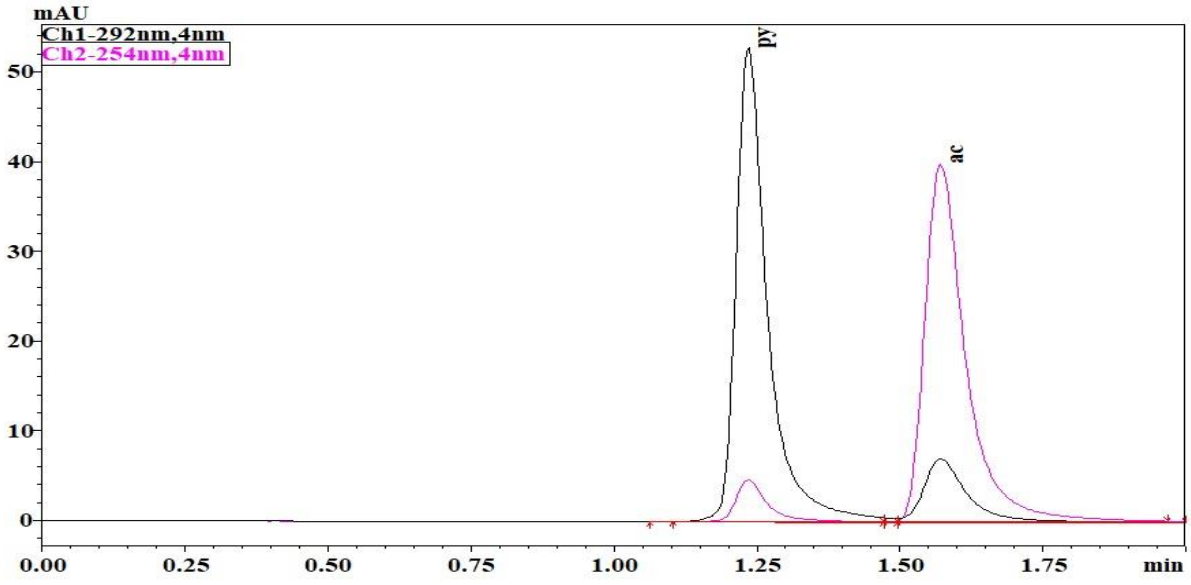

Figure 5: The chromatogram for separation of Acyclovir and Pyridoxine

System suitability parameters are shown in Table 3.

Table 3: system suitability parameter of the proposed UHPLC method

Parameter	Pyridoxine	Acyclovir
Retention time (min.)	1.24	1.57
Resolution*	————	2.89
Tailing factor	1.1	1.2
Column effeciency (NO. of theoretical plates)	2345	2520
HETP (µm)	21.324	19.843
capacity factor k'	5.194	6.853
selectivity α	————	1.319

Confirmation of Peak purity

Confirmation of peak identity and purity was achieved using the PDA and MS detectors of the instrument. The identity was confirmed by the full MS scan of the analytes' peaks and the 3D view of the chromatogram. Where peaks' purity was confirmed by the purity curve. Data are shown in Figure 6.

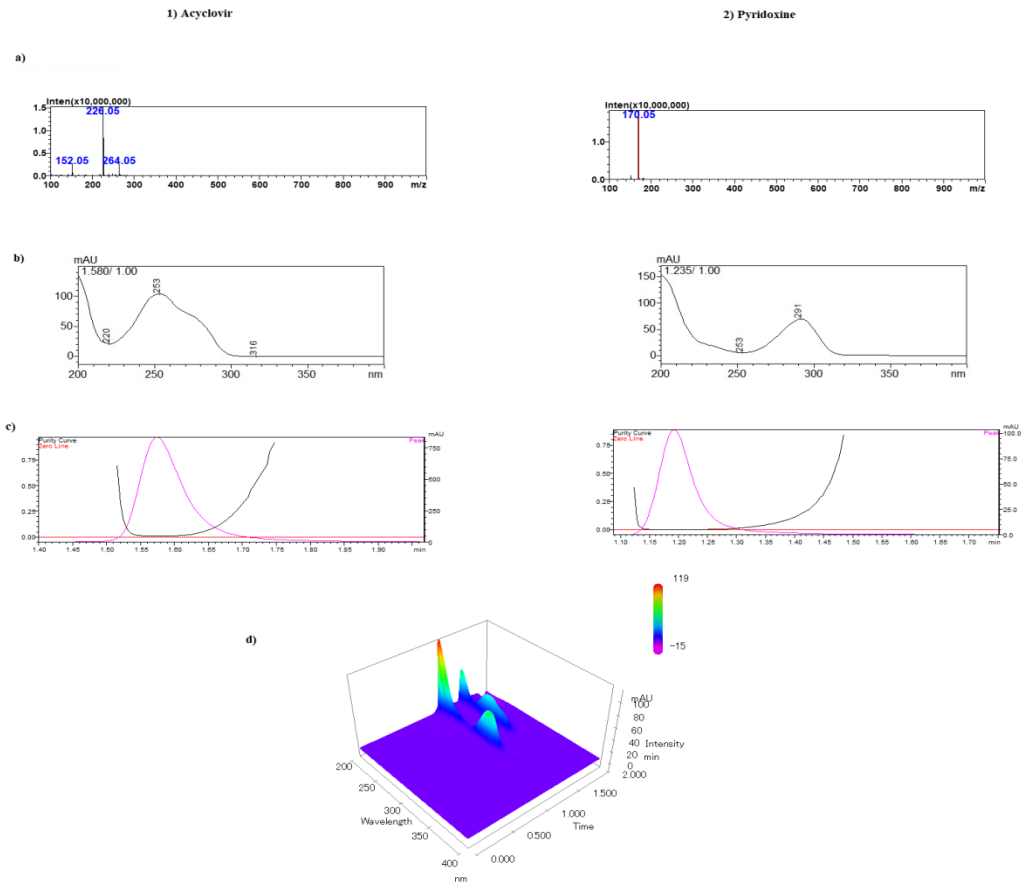

Figure 6: peaks' purity confirmed by the purity curve

The effect of the absorbed dose of gamma sterilization on the degradation of Acyclovir with or without Pyridoxine

Different solutions of Acyclovir with or without the free radicle scavenger Pyridoxine were prepared with concentration 100 µg/mL Acyclovir and 200 µg/mL Pyridoxine. The prepared solutions were exposed to different doses of gamma irradiation. Then, the stability of Acyclovir towards gamma sterilization was assessed by the developed UHPLC method. In the tubes where Pyridoxine is absent and only Acyclovir present, it was found that Acyclovir was almost completely degraded even with the least used dose of gamma irradiation (10 kGy). On the other side, in the tubes where Pyridoxine is present with Acyclovir, it was found that Acyclovir was partially protected from degradation even with the largest used dose of gamma irradiation (50 kGy). At 25 kGy dose, which was used for the sterilization of the ophthalmic hydrogel formulation, Pyridoxine protected 50 % of Acyclovir from degradation. All the data are presented in Table 4.

Validation

According to ICH criteria, the validated UHPLC procedure was carried out. When it comes to linearity, accuracy, precision, LOQ, and LOD (26). Table 5 lists all the validation parameter results.

Statistical comparison

According to Table 6, there were no statistically significant differences between the suggested technique's results and those obtained by using the reference method when they were compared.

Conclusion

The developed and validated UHPLC method could separate Acyclovir from the free radicle scavenger Pyridoxine with perfect resolution and in short run time. Quality by Design strategy was utilized to assess the main and interaction effects of the studied factors with the least number of runs. The results obtained from the developed UHPLC method proved that vitamin B6 (Pyridoxine) protected Acyclovir from complete degradation during the gamma sterilization step. The developed method proved to be accurate, precise, and selective where it could be used in quality control laboratories.

Author Contributions

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