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Spectrophotometric method for determination of Glucosamine Sulphate in dosage forms by derivatization using Quality by Design approach

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Abstract

A Quality by Design approach was used to develop a sensitive spectrophotometric method for determination of glucosamine Sulphate in pharmaceutical dosage forms and optimize a derivatization reaction of glucosamine with o-phthalaldhyde. This strategy was focused on derivatization process understanding and analytical method development and validation. Both of them were evaluated by applying risk assessment and Design Space tools. The scouting step was carried out by analyzing different derivatizing agents, type of buffers, pH of buffers, and time of reaction. Five critical process parameters (CPPs) were selected related to, pH of borate buffer, volume of 2-mercaptoethanol (ME), volume of o-phthalaldhyde (OPA), and the time of reaction. The effect of different levels of CPPs on critical quality attributes (CQAs) (the absorbance of derivative) was evaluated in the screening design using fractional factorial design. Using response surface methodology and contour plots the optimum values of the selected factors were determined and the design space of the method was defined. Finally, control strategy was designed and the method was validated and applied to a real sample of glucosamine dosage forms using the optimum derivatization conditions of the reaction; pH of borate buffer 10.5, concentration of buffer 0.05M, Time of reaction 10.7 minutes, volume of ME 100µL, and volume of OPA 5.1mL at room temperature. The results of the present study clearly shown that the developed method was sensitive relative to the other published spectrophotometric and HPLC methods with identification of the most significant factors affecting the derivatization reaction.

Keywords: Glucosamine Sulphate; spectrophotometric method; Derivatization, Experimental design; Design space.

1. Introduction

Glucosamine sulphate (GLS) is commonly used as a therapeutic agent for treatment of osteoarthritis, although its acceptance as a medical therapy varies. It is an amino sugar and a prominent precursor in the biochemical synthesis of glycosylated proteins and lipids. Since glucosamine is a precursor for glycosaminoglycan and glycosaminoglycan is a major component of joint cartilage, and supplemental glucosamine may help to rebuild cartilage and treat arthritis. (Sweetman, 2007)

Different methods of analysis were published for determination of GLS as spectrophotometric methods (Wu et al., 2005; Gaonkar et al., 2006) , HPLC methods, (Kumar et al., 2014; Song et al., 2012) HPTLC methods (Sullivan, et al., 2005; Sam Solomon, et al., 2010) and electrophoresis methods (8) (Vaclavikova et al., 2013).

Quality by Design (QbD) has become an important paradigm in the pharmaceutical industry since its introduction by the US Food and Drug Administration. The goal of QbD is to build quality into pharmaceutical products to ultimately protect patient safety. It is possible to apply QbD principles to the development of the analytical method itself. This approach can lead to better analytical methods, and can positively impact the broader QbD aspects of method development (ICH, 2009; ICH, 2005).

Method development using the principles of QbD emphasizes a systematic approach and applying scientific understanding and risk management approaches. Two key concepts can be introduced that aid in implementation of QbD. The first concept is design space and ICH defines design space as an “established multidimensional combination and interaction of material attributes and/or process parameters demonstrated to provide assurance of quality (Orlandin et al., 2014; Sun et al., 2010; Rozet et al., 2013). Understanding the design space for a pharmaceutical process generally involves the identification of critical attributes for the input materials and the process. By using this definition, changes within the design space of the method are not considered to be a change to the method. The second QbD concept is control strategy. The control strategy is obtained from the process understanding gained from modeling the design space and its purpose is to ensure the final quality of the method (Huang et al., 2009).

In this presented work, a sensitive, rapid spectrophotometric method was developed for determination of GLS by derivatization using o-phthaladehyde and by implementing the key elements of QbD to study the factors affecting the derivatization process and the performance of the method.

Glucosamine sulphate (GLS) is commonly used as a therapeutic agent for treatment of osteoarthritis, although its acceptance as a medical therapy varies. It is an amino sugar and a prominent precursor in the biochemical synthesis of glycosylated proteins and lipids. Since glucosamine is a precursor for glycosaminoglycan and glycosaminoglycan is a major component of joint cartilage, and supplemental glucosamine may help to rebuild cartilage and treat arthritis. (1)

Different methods of analysis were published for determination of GLS as spectrophotometric methods (2-3), HPLC methods (4-5), HPTLC methods (6, 7), and electrophoresis methods (8, 9).

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method. The second QbD concept is control strategy. The control strategy is obtained from the process understanding gained from modeling the design space and its purpose is to ensure the final quality of the method. (15)

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2. Materials and Methods

2.1.Instrumentation

A double-beam Shimadzu (Japan) UV-VIS Spectrophotometer (UV-1601 PC), model TCC-240 A. The bundled software is UVPC personal spectroscopy software version 3.7 (Shimadzu). The spectral bandwidth is 2 nm and the wavelength scanning speed was 2800.0 nmmin-1.

2.2.Materials and Reagents

All experiments were performed using standard of Glucosamine Sulphate was purchased from AUG Pharm. and certified to contain 99.99(w/w). Juflex® Capsules (EMA Pharm manufactured by Pharaonia Pharmaceuticals Cairo, Egypt with Batch no. 9092004 Claimed to contain 500mg per capsule), and Glucosamine® Capsules (EVA Pharm, Cairo, Egypt were purchased from local pharmacies. Borax, OPA, 2- ME sodium hydroxide, hydrochloric and phosphoric acids and Methanol, were obtained from Sigma Aldrich (Germany).

2.3. Derivatization conditions

The optimum condition of derivatization of Glucosamine Sulphate was derived from the Minitab software which consisted of 0.05M Borate buffer (pH 10.5), 100µL ME with 5.1mL o-phthalaldhyde solution and 10.5 minutes as time of reaction for formation of Isoindole derivative.

2.4. Standard solutions

A stock solution of Glucosamine Sulphate was prepared by dissolving Glucosamine Sulphate in water to obtain a concentration of 1000µg/ ml. Calibration curves were established in a range of 10-90µg/mL. Borate buffer was prepared by dissolving 19.07 gm Borax in water to obtain 1000 mL of 0.05M borate buffer and the pH of the buffer was adjusted using sodium hydroxide and hydrochloric acid.

OPA solution was prepared before use by dissolving OPA in 1.25 mL methanol in dark then adding 90mL borate buffer and 2-ME and leave in dark for 1 hour.

2.5. Method development

Method development was done by using the concept of QbD and DOE. The mains effects, interactions between variables were calculated to study their effects on the formation of derivative and performance of the method and to derive the optimum conditions of the reactions by applying minimum numbers of experiments.

2.6. Method Validation

2.6.1. Linearity and range

Different aliquots of Glucosamine Sulphate standard solution were transferred into a series of 25mL volumetric flask and should be covered with aluminum foil to be protected from light; each was mixed with 5.7mL OPA buffered solution. The mixture was left for 10.5 minutes, and then the volume was completed to 25mL with distilled water. The absorbance was recorded at 340nm and plotted against its corresponding concentration and the regression equations were computed.

2.6.2. LOD & LOQ

The limit of detection (LOD) and limit of quantification (LOQ) were calculated according to the guidelines to test the limits of the proposed method.

2.6.3. Accuracy

The accuracy study was demonstrated by using the standard addition technique. This technique was applied by adding different concentrations within the calibration range (30, 50, 70 µg/mL).to a fixed concentration from the dosage form. The mixtures were analyzed and the mean percentage recoveries and standard deviation results were obtained for Glucosamine sulphate.

2.6.4. Precision

Repeatability of the method was evaluated by determining three different concentrations for compound and analyzing it by the same procedure under experimental section and they were repeated three times within a day (intra-day precision). While intermediate precision was evaluated by analyzing the three concentration levels three times on three consecutive days (inter day precision). The mean and relative

standard deviation values for Glucosamine Sulphate were calculated.

2.6.5. Robustness

To evaluate simultaneous variations of the factors on the considered responses, the experimental design is recommended for robustness testing. The experimental design was performed to evaluate the behavior of the response around the nominal values of the factors. The fractional factorial design was used to test the robustness of the method by changing the levels of factors (pH of buffer, concentration of buffer, time of reaction, volume of 2-ME and volume of OPA).

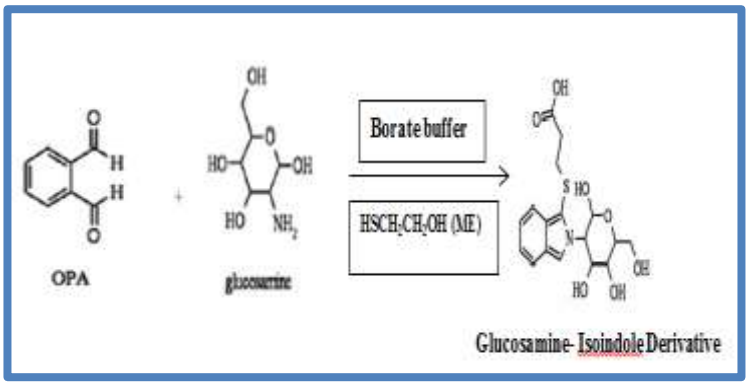
2.6.6. Pharmaceutical dosage form

Capsules of each pharmaceutical product were separately weighed. A portion of the powder of each pharmaceutical product equivalent to 100 mg of Glucosamine Sulphate was accurately weighed, separately transferred to 100 ml volumetric flasks and dissolved in about 50 ml methanol using ultrasonic bath (15 min) and cooled to room temperature. The solutions were diluted to volume with the same solvent and then filtered through 0.45 µm membrane filters (Millipore, Milford, MA). The first portions of the filtrates were discarded and the remainders were used as stock sample solutions. Further dilutions of the prepared solutions were carried out using distilled water to reach the linearity range specified for drug in the calibration curves. The general procedures described under calibration were followed and the concentration of each drug was calculated from the corresponding regression equations and the percentages recoveries were then calculated.

3. Results & discussion

The Glucosamine is weakly absorbing in UV region as shown in figure (1). Hence, conventional spectrophotometric method cannot be applied for its determination. The proposed method depends on derivatization of Glucosamine after conversion of its primary amine by OPA buffered solution in basic medium using ME to form glucosamine-Isoindole derivative (Scheme 1). The absorbance of the derivative product was measured at 340nm as shown in the overlay spectra (1).

Scheme 1: Chemical reaction of OPA & ME with primary amine containg drugs as Glucosamine



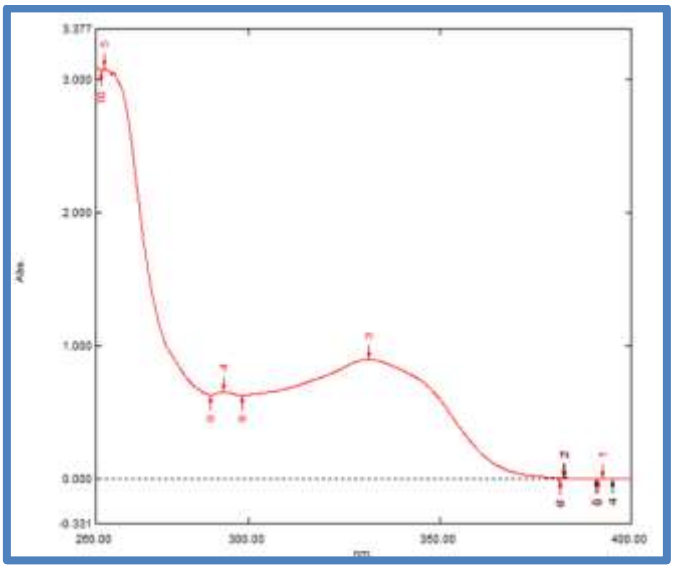


Figure (1): UV absorption spectra of Glucosamine Sulphate of 30µg/mL (…….) and glucosamine-isoindole derivative of 30µg/mL (————) in distilled water.

3.1. Analytical target profile

The analytical target profile was defined as development of spectrophotometric method for determination of Glucosamine Sulphate in bulk and in dosage form by derivatization. The performance of the method was evaluated by measuring the stability of the derivative. The scouting step should be applied to reach the ATP by analyzing different factors affecting the derivatization. The obtained spectrum pointed out the use of buffers of high pH to allow the formation of derivative. But during the scouting phase, there was non-significant effect of temperature. So, the starting point for screening to

reach the knowledge space based on using borate buffer in pH range (8.5-10.5) in concentration between (0.03-0.07M), time of reaction from 5 minutes to 10 minutes and volumes of ME and OPA in ranges (100-200µL) and (1.5-4.5mL) respectively at room temperature.

3.2. Critical quality attributes, critical process parameters, and quality risk assessment

The critical quality attributes (CQA) are responses, which are used to investigate the method performance. CQA of spectrophotometric method was formation of derivative which influences the performance of the method.

From literature and Ishikawa diagram as shown in **figure (2)**, the critical process parameters (CPP) that should be selected, studied and controlled to evaluate the method performance were pH of buffer, concentration of buffer, time of reaction, volume of ME, and OPA volume.

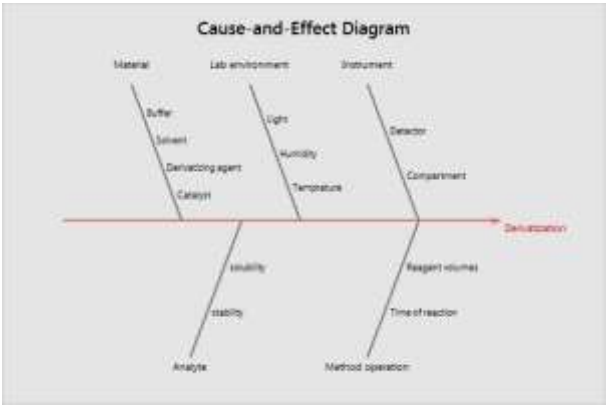


Figure (2): Ishikawa diagram for identification of critical process parameter

3.3. Design of experiments and knowledge space

The concept of DOE was used to evaluate the factors, the interaction between them, and screen these factors by using the fractional factorial design (2^{4-1}) as shown in **table (1)**. From this design, it can be concluded that the 3 factors and model curvature were significant since the p value ≤ 0.05 while the volume of ME and concentration of buffer were non-significant, this imply that RSM should be performed by using central composite design. By using the Minitab software, the response of each factor was evaluated according to the absorbance of the derivative product as presented in main effects plots in **figure (3)** which indicated the most main effect of the response was the time of reaction. There was a complete interaction between the factors and should be put in consideration which level will be used for each factor while there was no interaction between the time of reaction and ME as shown in interaction plots in **figure (4)** and the coded and uncoded values in the design presented in **table (2)**.

From screening design, the ANOVA and model fit statistics indicate significant model with p value0.007 and adjusted R^2 0.9908 which indicate the fitness of the model.

Pareto charts evaluated the ranking of the factors affecting the formation of the derivative from time of reaction; the most significant factor to ME (the least non-significant factor) on the method performance as shown in **figure (5)**. So RSM approach was used to reach the optimum conditions for derivatization by using desirability functions and to define the design space.

The equations of models derived from Minitab software were

Y (Absorbance) = 0.72+ 0.108pH- 0.0031buffer conc. + 0.164Time of reaction – 0.13 Volume of ME+ 0.11 OPA volume+0.015buffer conc.*Rx time + 0.0016 buffer conc.*OPA.

3.4. Response surface methodology and design space

The factors to be further studied by CCD were pH of phosphate buffer (7.7-11.3), time of reaction (2.5-15minutes), and OPA volume (0.3-5.7), while the other factors would be in their nominal values. For estimation of coefficients, 20 runs in CCD was planned including 7 runs of center point to evaluate the experimental error and put into consideration the interactions between variables. The optimization design was reported in **table (3)** with the measured responses.

The polynomial quadratic equations are

Y(Absorbance)=0.6319+ 0.1271pH+ 0.1273 Rx time+ 0.1534 OPA vol.+ 0.0252 pH*pH+ 0.0285 Rx time*Rx time- 0.0080 OPA vol.*OPA vol.- 0.0060 pH*Rx time+ 0.0365 pH*OPA vol.+ 0.0140 Rx time*OPA vol.

In optimization design as in **table (4)**, the ANOVA and model fit statistics investigate the validity of the model. By applying CCD, we can predict the optimization of the method to search along response surface for optimal range of factors to satisfy a goal such as maximize the absorbance of derivative.

To reach CQA of the proposed method by getting absorbance of the derivative between (1-1.5), the optimizer was used in the Minitab software to search for the global solution as shown in **figure (6)**.

Table (1): Screening of knowledge space: fractional factorial design

pH	buffer Conc.	Rx. time	Mercptoethanol Vol.	OPA Vol.	Absorbance at 340nm
-1	+1	+1	-1	-1	0.600
+1	+1	-1	+1	-1	0.769
+1	-1	+1	-1	+1	1.350
0	0	0	0	0	0.916
0	0	0	0	0	0.964
0	0	0	0	0	0.937
+1	-1	-1	-1	-1	0.783
+1	+1	+1	+1	+1	1.326
-1	-1	+1	+1	-1	0.757
-1	+1	-1	-1	+1	0.710
-1	-1	-1	+1	+1	0.746

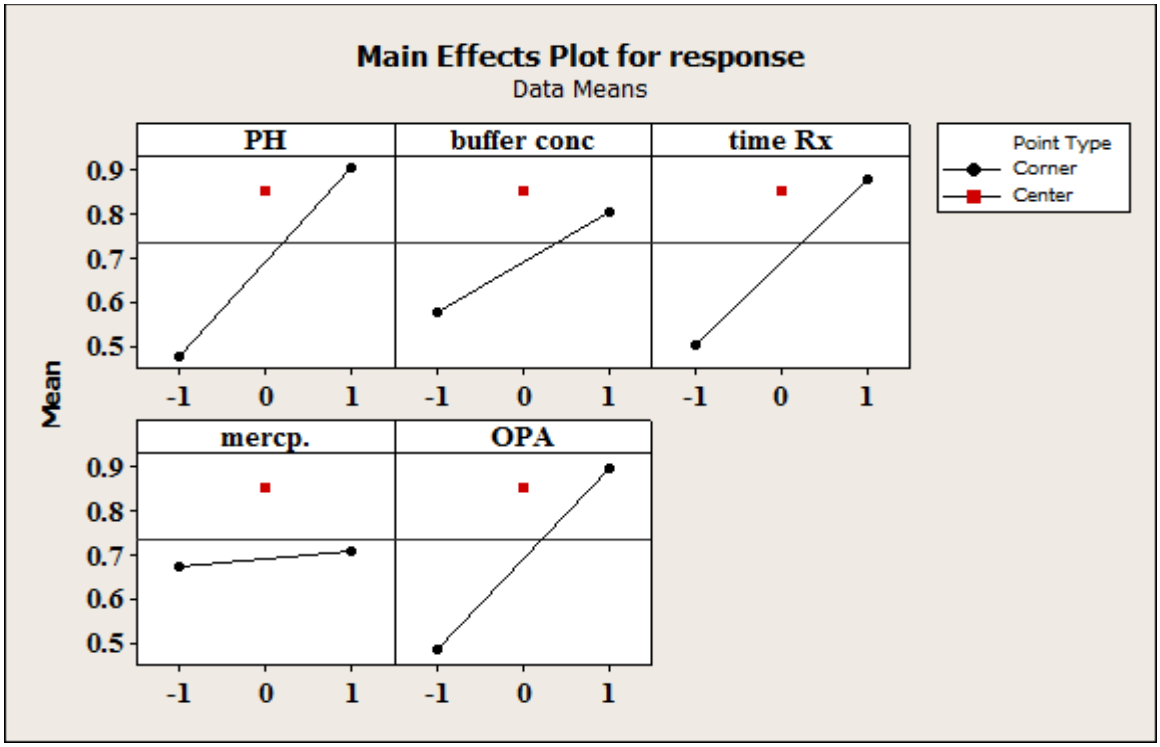


Figure (3): Main effect plots for derivatization reaction

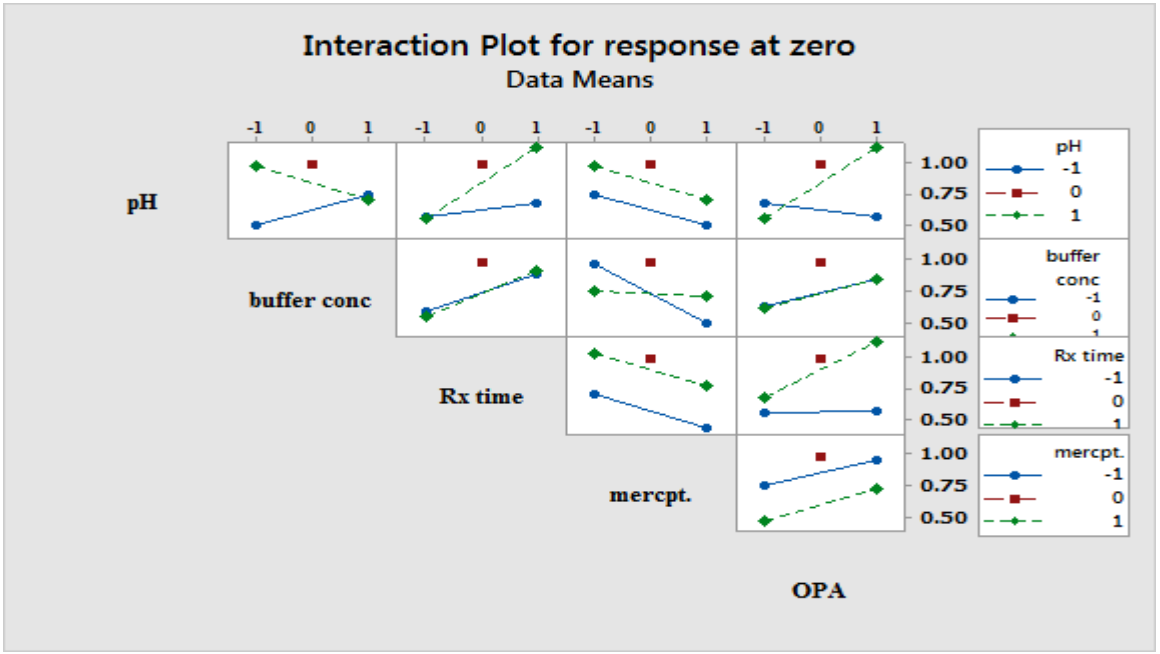


Figure (4): Interaction plots of parameters and their effect on the response

Table (2): The coded and uncoded values

Factors	Levels				
	-1.8	-1	0	+1	+1.8
pH of borate buffer	7.7	8.5	9.5	10.5	11.3
Buffer conc.	-	0.03M	0.05M	0.07M	-
Time of Rx	3min	5min	7.5min	10min	12min
ME volume	-	100µL	150µL	200µL	
OPA Vol.	0.3ml	1.5ml	3ml	4.5ml	5.7ml

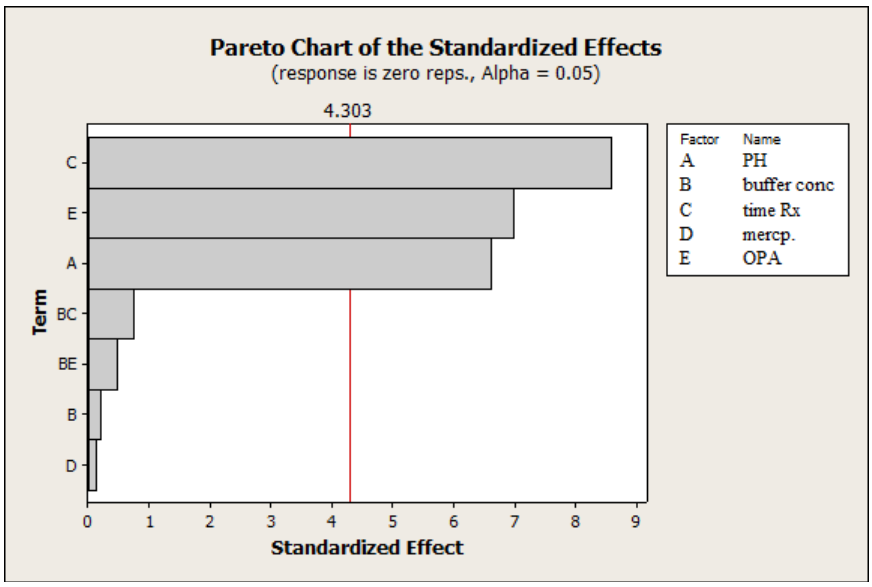


Figure (5): Pareto charts of the parameters and their effects on the response

Table (3): Response surface methodology: central composite design

Run	pH	Rx. time	OPA Vol.
1	-1.0	1.0	-1.0
2	0.0	1.8	0.0
3	0.0	0.0	0.0
4	0.0	0.0	-1.8
5	0.0	0.0	0.0
6	1.0	1.0	1.0
7	0.0	0.0	0.0
8	1.0	-1.0	1.0
9	1.8	0.0	0.0
10	0.0	0.0	0.0
11	0.0	0.0	0.0
12	1.0	1.0	-1.0
13	-1.0	1.0	1.0
14	-1.8	0.0	0.0
15	0.0	-1.8	0.0
16	-1.0	-1.0	1.0
17	-1.0	-1.0	-1.0
18	0.0	0.0	1.8
19	0.0	0.0	0.0
20	1.0	-1.0	-1.0

Table (4): A summary list ANOVA results in optimization design

Item	Response
P value	0.002
R Squared	0.940
R Adjusted	0.890
R Predicted	0.570

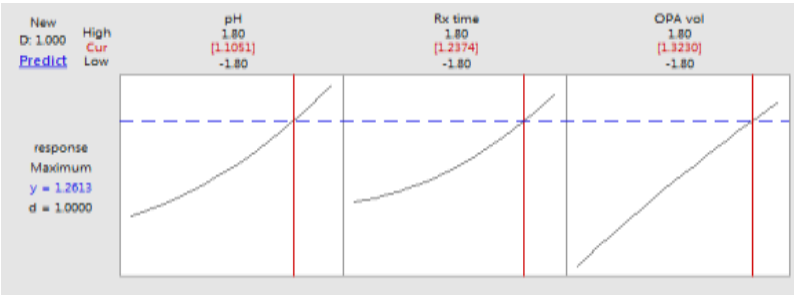


Figure (6): Desirability plot for Derivatization optimum conditions .

In this study, the DS calculated from the original set point that derived from response optimizer [pH of borate buffer 10.5, concentration of buffer 0.05M, Time of reaction 10.7minutes, volume of ME 100µL, and volume of OPA 5.1mL at room temperature to obtain the desirability of derivative formation more than 1. As shown in contour plot in **figure (7)**, the design space colored in white corresponding to the following values: pH of borate buffer (9-.11.3), and OPA volume (4.5-5.7) with time of reaction (9.5-12 minutes)

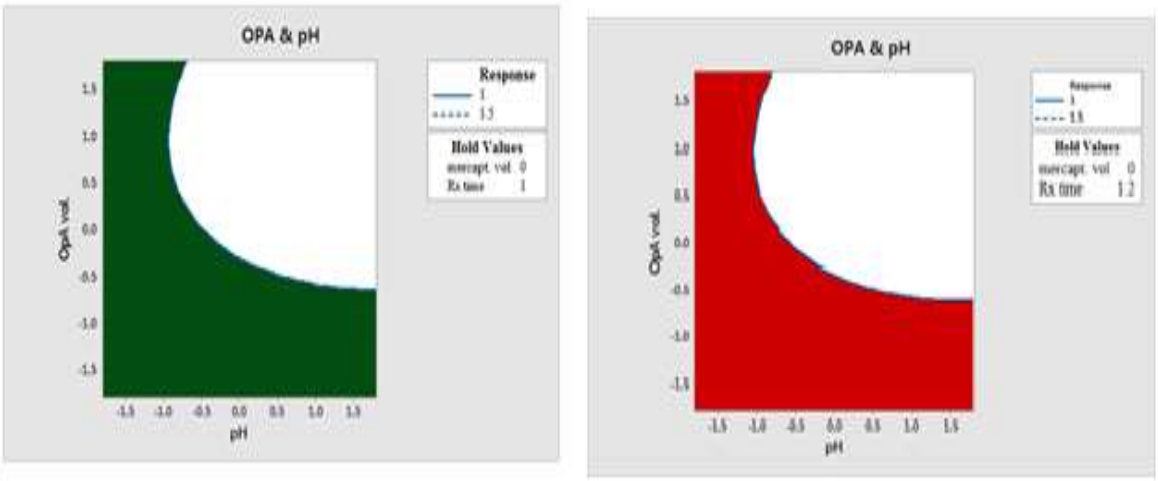


Figure (7): Overlay contour plots between factors to maximize the derivative formation

3.5. Robustness:

Inside the DS each point can be used as working point. After application of these working conditions as robustness study, the formation of Isoindole derivative was obtained with a reasonable stability. The ranges examined inside the DS around the optimum conditions of the method (pH of borate buffer 10.5, concentration of buffer 0.05M, time of reaction 10.5minutes, volume of ME 100µL, and volume of OPA 5.1mL) and the corresponding response were observed as shown in **table (5)**. So, to achieve the robustness step, the p values of all parameters factors were analyzed which proved their non-significant effect on CQAs as their values above 0.05 as shown in **table (6)**.

3.6. Method Control3

Control strategy was designed, in order to assure that the method is performing as intended. The control strategy presented as key points the need of control in derivative formation evidenced during robustness study. The lower and the higher values of the CQAs measured during system study were chosen as limits for establishing a range of accepted CQA value, and corresponded to the following ranges: absorbance of isoindole derivative (1-1.5).

3.7. Method validation and application to dosage form:

3.7.1. Range &Linearity: The linearity of the method was demonstrated in concentration range of 10-90µg/mL for Glucosamine Sulphate. The linear regression analysis was performed as mentioned in the experimental conditions and the regression parameters were calculated as shown in table (7). The correlation coefficient was found 0.9997.

3.7.2. LOD and LOQ

LOD and LOQ were determined by calculating first the standard deviations of residuals of concentrations. This statistics method was effective method to detect these items relative to other methods to test the sensitivity of the method. As shown in **Table (7)** by using these rules:

LOD = 3.3σ /S and LOQ = 10σ /S

3.7.3. Precision

Repeatability of the method was evaluated by selecting three different concentrations for Glucosamine Sulphate and analysing them and they were repeated three times within a day (intra-day precision) and its relative standard deviation was 1.22. While intermediate precision was tested by analysing the three concentration levels three times on three consecutive days (inter day precision) with relative standard deviation 0.42**.** From The mean recovery and relative standard deviation values as presented in **table (8)** and the

method was precise with relative standard deviation less than 2%.

3.7.4. Accuracy: The accuracy study was demonstrated by analyzing different concentrations of Glucosamine Sulphate Standard solution by Standard addition technique. The average percentage recovery at each concentration was determined and presented in **table (9)**. The results of mean percentage recovery and standard deviations were found 99.48±0.62 which proved that the method was accurate with satisfactory results.

Table (5): Robustness design fractional factorial design:

Exp. No.	pH	buffer conc.	Rx time	Mercptoethanol Vol.	OPA Vol.	Absorbance at 340nm
1	1	1	1	1	1	1.336
2	-1	1	-1	-1	1	1.345
3	0	0	0	0	0	1.37
4	-1	-1	-1	1	1	1.322
5	0	0	0	0	0	1.364
6	1	-1	-1	-1	-1	1.320
7	1	-1	1	-1	1	1.330
8	1	1	-1	1	-1	1.361
9	-1	1	1	-1	-1	1.320
10	-1	-1	1	1	-1	1.334
11	0	0	0	0	0	1.358

Table (6): The results of p-values in robustness design

Item	Response
Model	0.166
pH of buffer	0.265
Buffer Conc.	0.081
Rx time	0.241
Mercapto. Vol.	0.155
OPA Vol.	0.917
pH*Rx time	0.051
Buffer conc.*OPA vol.	0.917

Table (7): Regression and validation parameters for determination of Glucosamine sulphate by the proposed spectrophotometric method.

Parameters	Glucosamine sulphate
Linearity (µg/mL)	10-90
Intercept coefficient ± S.E	0.316±0.02
Intercept confidence interval	0.26-0.37
Slope coefficient± S.E	$0.019\pm2X10^{-4}$
Slope confidence interval	0.018-0.02
Correlation coefficient± S.E	0.9994
LOD (µg/mL)	2.6
LOQ (µg/mL)	7.8

Table (8): Intra-day and Inter-day precision for the analysis of Glucosamine Sulphate using the proposed HPLC method

sample	Intra-day		Inter-day
	Conc. (µg/mL)	Mean	Mean
Glucosamine Sulphate	10	101.5	101
	50	99.9	100.2
	90	99.1	100.8
	Mean± RSD	100.17±1.22	100.67±0.42

Table (9): Application of standard addition technique to the analysis of Glucosamine sulphate using the proposed spectrophotometric method

	% Recovery ± SD	Standard addition technique		
		Amount added (µg/mL)	Amount found	% Recovery
Juflex® capsule	100.46±0.43	10	9.94	99.4
		20	19.78	98.9
		30	30.04	100.1
		Mean± SD		99.47±0.60

3.7.5. Application

Application of the proposed method to pharmaceutical dosage form was demonstrated by preparing six replicate sample solutions for Juflex® capsules and Glucosamine® capsules. These samples were analyzed according to the proposed method and treated with OPA and ME in their optimum conditions and then percentage recoveries and standard deviation were calculated for Glucosamine sulphate as presented in **table (10)**. According to the results in the table, the method was valid to be applied to the dosage form with small standard deviation. The results obtained for the analysis of Glucosamine Sulphate in its dosage forms were statistically compared with those obtained by applying the reported method. The values of calculated t and F were less than the tabulated, which indicates that there was no significant difference was found between both methods as shown in **table (11)**.

Table (10): Determination of Glucosamine Sulphate in the pharmaceutical dosage forms by the proposed spectrophotometric method

Dosage Form	Conc. Of compounds	
	Joflex ® Capsules	Glucosamine® Capsules
	(30µg/ml)	(30µg/ml)
% Found	100.4	100.7
	100.3	100.6
	99.5	100.9
	100.1	100.2
	100.7	100.8
	99.1	100.7
Mean	100.08	100.65
SD	0.60	0.24

Table (11): Statistical comparison for results obtained by proposed method and HPLC reported method for the analysis of Glucosamine sulphate in Juflex® capsules

Items	Joflex ® Capsules	
	Proposed method	Reported method (2)*
Mean	100.08	100.09
SD	0.19	0.3
Variance	0.20	0.09
N	10	5
t-test	0.711	(2.446)[a]
F-test	2.38	(6.338)[a]

**It is USP method, mobile phase composed of (Methanol: Acetate buffer adjusted with acetic acid at pH= 5.9) (100:900) detection at 340nm at flow rate 1mL/min. using ODS (5mm x 3.3mm)
(a) theoretical values for t and F at p value = 0.05

4. Conclusion:

The derivatization of glucosamine with OPA by using the proposed method was sensitive compared to the published spectrophotometric methods (2-3) and HPLC published methods (5-8, and 10). The most significant factor affecting the stability of the glucosamine isoindole derivative was the time of reaction as the absorbance of the derivative starts to decline after 13 minutes. The proposed spectrophotometric method provides indirect method for the assay for glucosamine Sulphate in capsules. The method overcomes the problem of low absorptivity of Glucosamine in UV region. Also, the results presented the factors affecting the formation of Isoindole derivative and the effect of each factor on the formation of derivative by using design of experiments and quality by design.

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