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Optimization and Validation of Spectrophotometric Determination of Glucosamine in Dosage Form with Sodium 1,2-naphthoquinone-4-sulphonate

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Abstract

The present study developed rapid, sensitive and simple optimized method using spectrophotometric technique for the determination of Glucosamine (GL) in dosage forms. The method was performed based on the reaction of GL with sodium 1,2-naphthoquinone-4-sulphonate (NQS) in alkaline medium in order to form an orangey-colored product to be measured at 451 nm. Method optimization was essential as many experimental parameters can influence the derivatization process in terms of its efficiency and reproducibility, such parameters are the reaction time, reaction temperature, NQS concentration, alkali concentration and solvents. The current study aimed to optimize and validate a spectrophotometric method using the reaction of GL with NQS for determining GL in dosage forms. We identified and optimized the factors that may affect derivatization efficiency and reproducibility of the procedure. We further compared the data of the proposed method with a reference method, and statistical comparison showed ultimate agreement without significant differences in the accuracy and precision. The developed method is convenient, accurate and cost-effective for routine studies if compared to HPLC and other common techniques.

Keywords: Glucosamine; 1,2-naphthoquinone-4-sulphonate; pharmaceutical analysis; spectrophotometric determination.

1. Introduction

Several published methods described the determination of Glucosamine (GL) in dosage forms. These methods include spectrophotometry (Wu et al. 2005; Yamaguchi et al. 2004; Burtseva et al. 1986) , high - performance liquid chromatography (HPLC) (Hadad et al. 2012; Zang et al. 2006; Niu et al. 2006; Tekko et al. 2006; Eberendu et al. 2005; Zhou et al. 2005; Shen et al. 2005; Ji et al. 2005), TLC-densitometry (Esters et al. 2006; Sullivan et al. 2005), and capillary electrophoresis (Qi et al. 2006; Garcia and Henry. 2005).

Spectrophotometry is considered a simple and more convenient alternative technique. There is no peculiar chromophore is available on GL moiety for the direct analysis by UV-Vis absorption spectrophotometry. Therefore, it is difficult to develop an analytical spectrophotometric method for determination of GL due to lack of chromophoric group in the molecule. Thus, the use of UV or fluorescence detection was also excluded. Only three spectrophotometric methods have been reported for the determination of GL based on the reaction with ninhydrin (Wu et al. 2005), *O*-hydroxyhydroquinonephthalein and palladium (II) (Yamaguchi et al. 2004) and 4-dimethyl-aminobenzaldehyde (Burtseva et al. 1986).

Sodium 1,2-naphthoquinone-4-sulphonate (NQS) is a water soluble chromophore that forms colored derivative when it reacts with an alkaline medium containing a primary amino group (Zaitsu et al. 1999).

There is no reported studies involved an analytical method considering the reaction of the primary amino group of GL with NQS for the determination of GL. The current study was designed to optimize and validate a spectrophotometric method using the reaction of GL with NQS for determining GL in dosage forms. The derivatization process was optimized by identifying the factors that may affect the efficiency and reproducibility of this procedure. The method was applied successfully to quantify GL in dosage forms.

The developed method is direct, sensitive, accurate and highly cost-effective for routine studies when compared to HPLC (Hadad et al. 2012; Zang et al. 2006; Niu et al. 2006; Tekko et al. 2006; Eberendu et al. 2005; Zhou et al. 2005; Shen et al. 2005; Ji et al. 2005) and other techniques such as capillary electrophoresis (Qi et al. 2006; Garcia and Henry. 2005). However, other published spectrophotometric methods involved extraction of the chromophore into chloroform (Burtseva et al. 1986) and indirectly depend on fading of color complex (Yamaguchi et al. 2004). The calibration curve of GL in the spectrophotometric method based on reaction with ninhydrin (Wu et al. 2005) was linear between 40 to 400 µg ml^{-1}.

2. Experimental

2.1. Materials and reagents

Glucosamine sulphate, of a pharmaceutical grade, was used and approved purity of 99.8 %.NQS (Aldrich, Inc, St. Louis, USA) and Sodium hydroxide (Sigma-Aldrich, Inc, St. Louis, USA) were analytical grade. All the chemicals used the study were of analytical reagent grade. Commercial Joflex® capsule (Batch no 47505) used was produced by Pharaonia pharmaceuticals for EMApharm pharmaceuticals. Each capsule contains 500 mg of glucosamine sulphate. NQS solution was freshly prepared in water at concentration of 0.01M and protected from sunlight. A 0.1M aqueous NaOH solution was used. All solutions were prepared using distilled water.

2.2. Standard solutions preparation and calibration graph for GL

GL was prepared in water in a solution stock of (1 mg ml^{-1}). Distilled water was used to further dilute GL stock solution of in to working standard solution of 250 µg ml^{-1}**.** An aliquot of standard solution containing 40 -700 µg of GL was transferred to a 10 ml volumetric flask. 0.5 ml of 0.1 M NaOH and 2 ml of 0.01M NQS were added. The solutions were shaken and placed in a thermostatic oven at 60°C for 20 min. The reaction was discontinued by putting the test tubes in a bath of cold-water. The volume was then topped up with water. The absorbance of the generated color was measured at 451 nm against the corresponding reagent blank. For blank sample, GL solution was replaced with water. Calibration graph was constructed for GL standard solutions in concentration ranging from 4 to 70 µg ml^{-1} by plotting the absorbance against the corresponding concentration. A Linear relationship from the curve was obtained.

2.3. Optimization of derivatization conditions:

For a reproducible GL-NQS reaction, parameters that affect the reaction such as temperature, time, NQS concentration, alkali concentration and solvents were investigated and optimized. A working standard solution containing 40 µg ml^{-1} GL was used to probe the above factors. First, to examine the effect of temperature, derivatization was performed for 20 min using 2 ml of 0.01M NQS solution and 0.5 ml of

0.1M NaOH at 25, 40, 60, and 80 °C. Secondly, in order to test the impact of reaction time, derivatization was performed at 60 °C using 2ml of 0.01M NQS solution and 0.5 ml of 0.1M NaOH for 10, 20, and 30 min. Third, To investigate NQS concentration effect, the reaction was carried out at 60 °C for 20 min using 0.5 ml of 0.1M NaOH and various volumes of 0.01M NQS solution format a range of (0.5-5 ml). Last, to investigate alkali concentration effect, the reaction was performed at 60 °C for 20 min using 2 ml of 0.01M NQS solution and various volumes of 0.1M NaOH solution at a range of (0.5- 3 ml). The solutions were assayed according to the procedure previously described.

2.4. Pharmaceutical sample preparation

Twenty capsules were emptied, weighed and mixed. A 25 mg GL powder was exactly measured, transferred to volumetric flask (100 ml) and extracted with about 80 ml water using ultrasonic bath for 30 min then cooled to room temperature. A dilution step with 100 ml of water was followed. Then the solution was filtered using 0.45 µm membrane filters (Millipore, Milford, MA). The first fraction obtained from the filtrate was discarded whereas the consequent one was kept as a sample solution.

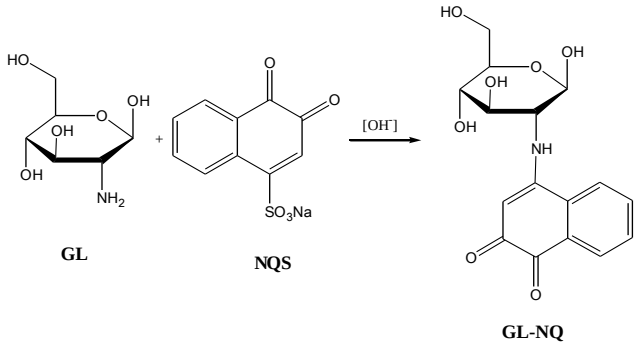
Sample solution was aliquoted, each of contained 40-700 µg of GL were transferred to a 10 ml volumetric flasks and derivatized as described Previously. The absorbance of formed solutions was measured at 451 nm against corresponding reagent blank.

3. Results and discussion

The developed method depends on the reaction of the primary amino group of GL with NQS in alkaline medium forming coloured GL-NQ derivative (**Scheme 1**) which measured its absorbance at 451 nm.

3.1. Optimization of derivatization:

The aim of the optimization process is to get a high reaction yield with a shortest reaction time possible, the factors affect the GL-NQS reaction is described in the following sections.



Scheme 1: The formation of GL-NQ derivative by reaction of GL with NQS.

3.1.1 Effect of reaction temperature

Figure 1a illustrates the impact of the derivatization temperature on the reaction progress at a fixed time of 20 min using 2 ml of 0.01 M NQS and 0.5 ml 0.1M NaOH solution**.** Using four reaction temperatures, the maximum yield was acquired at 60 °C. Accordingly, 60 °C was favored as the optimum reaction temperature, with a significant difference in yield enhancement when compared to the lower temperatures ($P < 0.05$).

3.1.2 Effect of reaction time

Derivatization was carried out at 60 °C using 2 ml of 0.01M NQS and 0.5 ml 0.1M NaOH solution for three time ponits10, 20 and 30 min. According to (**Figure 1b**), the highest yield was obtained after 30 min of reactionHowever, there was no significant difference ($P > 0.05$) detected after 20 min reactions. Therefore, 20 min was selected for our experiments.

3.1.3. Effect of NQS concentration

Different volumes of 0.01M NQS solutions starting from 0.5 to 5 ml were used for derivatization at 60 °C for 20 min using 0.5 ml 0.1M NaOH solution.

Data from (**Figure** 1**c**) indicates that there is no significant difference in the GL-NQ adduct yield between 2, 3 and 5 ml of 0.01 M NQS solutions. ($P > 0.05$). Consequently, 2 ml of 0.01 M NQS solution was favored as an efficient least concentration.

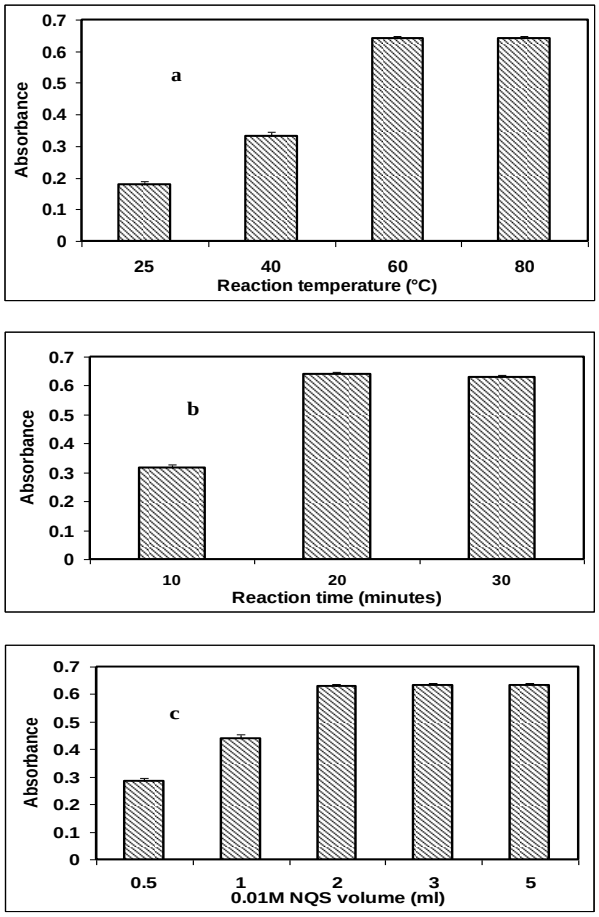


Figure 1: Optimization of reaction conditions for derivatization of GL using NQS. Effect of (A): temperature, (B): reaction time, (C): volume of 0.01M NQS on GL-NQ reaction yield (mean ± S.D., *n* = 3).

3.1.4 Effect of alkali concentration

Various volumes of 0.1 M NaOH were tried ranging from 0.5 to 3 ml and there was no significant difference in the GL-NQ adduct yield between 0.5 and 3 ml 0.1 M NaOH solution ($P > 0.05$). Therefore, 0.5 ml of 0.1M NaOH was chosen for all further studies.

3.1.5. Effect of solvent

Several solvents including water, methanol, ethanol, and acetonitrile were used for NQS reagent as well as for diluting the reaction mixture. Water produced highest absorbance values, thus it was the best solvent to be used.

Based on these findings, the derivatization reaction was performed at 60 °C for 20 min using 2 ml 0.01 M NQS solution and 0.5 ml of 0.1M NaOH; and water for dilution of the reaction mixture. The absorbance of the formed derivative was measured at 451 nm (**Figure 2**). The value of A (1%,1cm) of the obtained color was found to be 147.

3.2. Analysis of pharmaceutical product

The proposed spectrophotometric method was designed and to determine GL in capsules dosage forms. Seven replicates determinations were used and robust results were generated for GL , which match the label claims (**Table 1**).

Table 1: Determination of GL in commercial pharmaceutical product using the proposed spectrophotometric method.

Sample no.	GL conc. (µg ml^{-1})	% recovery
1	4	97.9
2	10	97.3
3	20	98.6
4	30	99
5	40	97.7
6	55	98.1
7	70	98.4
	Mean[a]	98.14
	S.D.[a]	0.57

[a] *Mean and S.D., percentage recovery from the label claim amount.*

Using the proposed method, calculated mean percentage of GL ± S.D. of seven replicates equals 98.14% ± 0.57. When our method was compared with previously published works, comparable results were obtained. The accuracy and precision of the proposed method was examined by determining Student's t-test and F-values. Both t- and F values did not exceed the theoretical values at 95% confidence, thus indicates there is no significant difference between the proposed method and previously validated ones (**Table 2**).

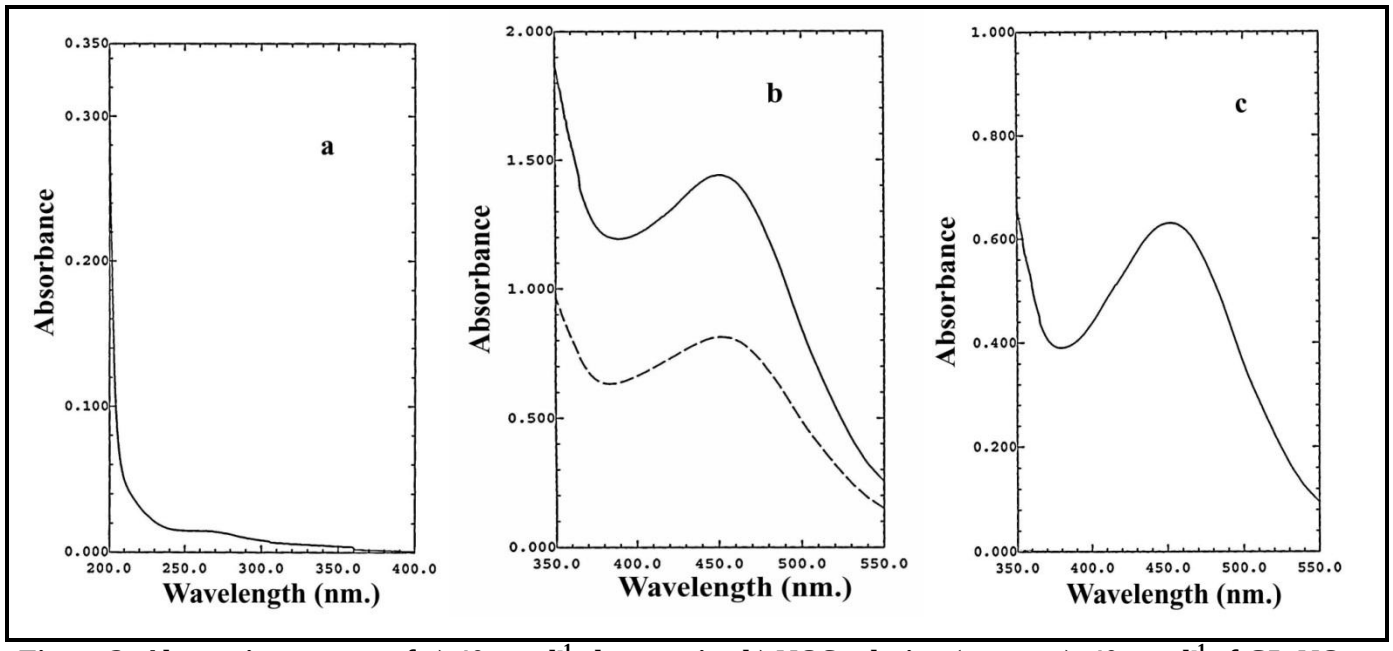


Figure 2: Absorption spectra of a) 40µg ml^{-1} glucosamine b) NQS solution (— — —), 40µg ml^{-1} of GL-NQ (————) against water. c) The reaction product of 40 µg ml^{-1} of GL with NQS against reagent blank.

Table 2: Statistical comparison between the proposed spectrophotometric method and the published method for determination of GL in Joflex® capsules.

	Mean ± S.D.[a]	
	Proposed method	Published method
Joflex® capsule	98.14 ± 0.57	98.49 ± 0.51
t	1.18	(2.18)[b]
F	1.24	(4.28)[b]

[a] Mean ± S.D., percentage recovery from the label claim amount, [b] Theoretical values for t and F at P = 0.05.

3.3. Validation of the method

3.3.1. Linearity

The linearity of the proposed method was evaluated by analyzing a series of different concentrations of GL. In this study, seven concentrations ranged between 4-70 µg ml^{-1}were evaluated. Each concentration was performed in to calculate absorbance variations between the samples. The linearity was evaluated from the high

Table 3: Characteristic parameters for the regression equation of the proposed spectrophotometric method for determination of GL.

Parameters	GL
Calibration range (µg ml^{-1})	4-70
Detection limit (µg ml^{-1})	2.70×10^{-2}
Quantitation limit (µg ml^{-1})	8.99×10^{-2}
Regression equation(Y)[a]: Slope (b)	1.47×10^{-2}
Standard deviation of the slope (S_b)	1.69×10^{-4}
Relative standard deviation of the slope (%)	1.15
Confidence limit of the slope [b]	1.45×10^{-2}- 1.48×10^{-2}
Intercept (a)	4.22×10^{-2}
Standard deviation of the intercept (S_a)	6.50×10^{-3}
Confidence limit of the intercept [b]	3.59×10^{-2}- 4.85×10^{-2}
Correlation coefficient (r)	0.9999
Standard error of estimation	3.63×10^{-3}

[a] Y = a+bC ,where C is the concentration of GL in µg ml^{-1} and Y is the absorbance, [b] 95% confidence limit.

value of the correlation coefficient and the intercept value. The difference was not statistically significant from zero (p = 0.05) different (**Table 3**). Using at least squares treatment of the results, the characteristic parameters for regression equation of the spectrophotometric method was obtained and listed in Table **3**.

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According to AMC (Analytical Methods Committee), a value of regression coefficient close to unity does not necessarily predict a linear relationship and the test for the Lack of Fit should be examined (Juan et al. 2005) (**Table 4**). The test examines the variance of the residual values (Draper and Smith 1981). The linearity was confirmed because calculated values were lower than the tabulated ones (α = 0.05).

3.3.2. Precision

Using three concentrations for GL, Precision, repeatability and intermediate precision were estimated at. One-way ANOVA statistical test was used to judge the data. .

Table 4. ANOVA (showing lack of fit calculation) for GL.

Source of variation	Sum of squares	degree of freedom	Mean sum of squares	F-Ratio
Total	0.698	21	0.033	2.41
Regression	0.697	2	0.349	
Residual	4.5×10^{-4}	19	2.37×10^{-5}	
Replicate	8.76×10^{-5}	7	1.25×10^{-5}	
Lack of fit	3.63×10^{-4}	12	3.02×10^{-5}	

The critical value of F-Ratio is 3.57 at α = 0.05

A design based on 8 days using 2 replicates was used. The results were statistical analyzed using the P-value of the F-test. Three univariate analyses of variance for each concentration level were made. Since the P-value of the F-test (**Table 5**) is always greater than 0.05, there was no statistical significant difference between the mean results obtained from one level of day to another at the 95 % confidence level.

3.3.3. Range

Practically, the required range, to give accurate, precise and linear results was used to establish the calibration. The calibration range of the proposed spectrophotometric method was given in **Table 3**.

3.3.4. Detection and quantitation limits

According to the International Conference on Harmonization (ICH). Recommendations (ICH, 1996), the approach based on the standard deviation (S.D.) of the response and the slope was used for determining the detection and quantitation limits. Table 3 illustrates the assessed theoretical values.

3.3.5. Accuracy

In order to study the effect of excipients in the pharmaceutical formulations, standard addition method was applied to GL-contained pharmaceutical formulation. Using six replicates, the mean percentage recoveries and standard deviation of the added GL were calculated (**Table 6**). The obtained results afforded good precision and accuracy. Thus, the excipients in exhibit no effect in the analysis of GL in its pharmaceutical.

Table 5: Analysis of variance for repeatability and intermediate precision for GL using the proposed spectrophotometric method.

Conc. Level	Sources of variance	Sum of squares	DF	MS	F-ratio	P-value
	Between	10.22	7	1.46	0.60	0.74
4 µg ml^{-1}	Within	19.33	8	2.42		
	Total	29.55	15			
	Between	14.69	7	2.10	2.08	0.16
35 µg ml^{-1}	Within	8.06	8	1.01		
	Total	22.75	15			
	Between	8.79	7	1.26	2.48	0.11
70 µg ml^{-1}	Within	4.06	8	0.51		
	Total	12.85	15			

Where DF is the degree of freedom and MS is the mean square. The critical value of F-ratio is 3.5 and P-value is 0.05.

3.3.6. Robustness

The effect of small variation in the experimental parameters e.g. temperature, time, NQS concentration and alkali concentration on the analytical performance of the method was used to estimate robustness. Variations in the parameters were as follows: reaction temperature by ± 2 °C, reaction time by ± 2 min., NQS concentration by ± 0.1 ml and alkali concentration by ± 0.1 ml. All differences show no significant effect on the performance of the method. Thus, confirmed that the proposed method is reliable during the routine applications.

3.3.7. Analytical Solutions Stability

In order to avoid delays during analysis, stability of all the solution was tested. Starting with the stability of NQ derivative of GL in water, we confirmed all samples were stable for at least 3 hour at room temperature. A stability of one week 4°C was confirmed for GL stock solution. Stability of 0.01 M NQS solution in water was examined and the solution was stable when protected from light at room temperature, for at least 10 h.

Table 6: Accuracy of the proposed spectrophotometric method determined by the recovery of GL from dosage form.

Exp. No	Claimed conc. (µgml^{-1})	Added conc. (µgml^{-1})	% Recovery of added
1	30	5	98.6
2	15	10	99.3
3	20	20	98.6
4	5	30	100.3
5	7	8	99.2
6	10	60	99.8
		Mean	99.30
		S.D.	0.67

4. Conclusion

An optimized spectrophotometric derivatization method to determine of GL in dosage forms has been developed and validated. The method is relatively simple and rapid to implement. It requires one step derivatization prior to measuring absorbance. The method showed high precision and accuracy as well as it is robust for the routine quantitation of GL in dosage form.

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