ejpmr, 2018,5(4), 118-129

SJIF Impact Factor 4.897

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

VALIDATION OF A NON-OFFICIAL ANALYSIS METHOD FOR A GRANULATED PRODUCT BASED ON GLUCOSAMINE AND CHONDROITIN

Lester Rojas-Quesada[1], German L. Madrigal-Redondo[2]*, Rolando Vargas-Zuñiga[2] and Santiago Rodríguez-Sibaja[1]

[1]School of Pharmacy, Universidad Latina de Costa Rica, San Pedro, San José, Costa Rica.
[2]Biopharmaceutics and Pharmacokinetics Laboratory (LABIOFAR), Pharmaceutical Research Institute (INIFAR), Faculty of Pharmacy, Universidad de Costa Rica, San José, Costa Rica.

***Corresponding Author: Dr. German L. Madrigal-Redondo**

Central Sterilization Service, Mohamed V Military Teaching Hospital, Rabat Morocco.

Article Received on 07/01/2018 **Article Revised on 27/02/2018** **Article Accepted on 19/03/2018**

ABSTRACT

Introduction: Glucosamine and chondroitin are structural constituents of the extracellular matrix of articular cartilage; they provide the cartilage with its mechanical and elastic properties for its ability to retain water. These are widely used today for the treatment of osteoarthritis worldwide and are classified as Symptomatic Slow Action Drugs for Osteoarthritis (SYSADOA). **Objective:** To develop and validate a non-official method of analysis for the assay of a product based on glucosamine and chondroitin in the form of granules. **Method:** A High-resolution liquid chromatography (HPLC) method was developed and validated, emphasizing that there is currently no other official HPLC method for the analysis of these active ingredients. The procedure used a C18 chromatographic column at a mobile phase flow of 1 mL / min and using an injection volume of 10 µL. The active ingredients of the analyzed product are in the form of sodium hydrochloride for glucosamine and sodium sulfate for chondroitin. **Results:** The results obtained for the analysis of each validation performance parameter met the established acceptance criteria. **Conclusions:** By accepting each of the performance parameters for the developed method, it can be concluded that it is duly validated and can be reliably used.

KEYWORDS: Glucosamine, chondroitin, osteoarthritis, high performance liquid chromatography, analysis method, validation.

INTRODUCTION

Osteoarthritis is a common disease and is the most important cause of disability among the elderly; until recently, the treatment of osteoarthritis was based on the administration of NSAIDs, which rapidly improve painful symptoms, but these are not able to modify the evolution of the disease, since the symptoms reappear after the suppression of treatment. In addition, they are not exempt from security problems. (Abad Santos, Ochoa Mazarro, & García García, 2011).

It has been shown that some compounds, known as SYSADOA (Symptomatic Slow Action Drugs for Osteoarthritis) can produce beneficial effects on the articular cartilage, presenting a global efficacy similar to NSAIDs (Non-Steroidal Anti-inflammatory Drugs). However, its effect takes longer to reach and persists for a few months after the suppression of treatment. This group includes drugs such as hyaluronic acid administered intra-articularly and oral glucosamine and chondroitin. (Abad Santos, Ochoa Mazarro, & García García, 2011).

Chondroitin sulfate is a natural copolymer based mainly on the two disaccharides obtained from the cartilage of terrestrial and marine living beings. Depending on the animal species, it shows different proportions of 4-sulfate and 6-sulfate groups. It is a white or almost white hygroscopic powder, very soluble in water; practically insoluble in alcohol and acetone. In addition, a 5% solution in water has a pH of 5.5 to 7.5. (Sweetman, 2009).

Glucosamine is a natural substance found in chitin, mucoproteins and mucopolysaccharides. It is involved in the glycosaminoglycan formation, which forms the cartilage tissue in the body and it is present in tendons and ligaments. In addition, glucosamine in its hydrochloride form has a pH of 3 to 5 in a 2% solution in water and is sensitive to light and heat. (Sweetman, 2009).

Regarding the analytical methodology, only official methods are found for this product, which only contemplate the test of the product in capsule and tablet form, as well as requiring an analysis for each active ingredient separately.

A specific adaptation of an analytical technique was carried out for the product analysis of both glucosamine and chondroitin under the same method and the validation was carried out for each of the active ingredients separately; it should be noted that there is no HPLC method of analysis for these components.

METHODS

The analysis method developed was carried out using a high-resolution liquid chromatography (HPLC) analysis. Chromatographic conditions include a mobile phase composed of acetonitrile and an aqueous solution in a 90/10 ratio respectively. The aqueous medium consists of phosphoric acid (0.8mL) and octanesulfonic acid (1.2g) in 900mL of water. In addition, a flow rate of 1.0 mL/min, an injection volume of 10 µL, a C18 chromatographic column and UV detection at a wavelength of 195 nm.

Unconventional standards of glucosamine hydrochloride and chondroitin sulfate at 98.4% and 96.7% were used, respectively, as reference standards. The standard solutions of glucosamine 100% were prepared looking for a final concentration of 400 ug/L, while for the standard solutions of chondroitin a final concentration of 350 ug/L was reached.

First, each standard was weighed separately and a dilution was made to obtain a concentration of 2000 ug/L for the standard glucosamine solution and 1750 ug/L for the standard chondroitin solution. Subsequently, an aliquot of each prepared standard solution was taken to form a mixture of standards and thus obtain the final concentration of the standards, described above.

For samples preparation, an equivalent amount was weighed against 1500 mg of glucosamine in base form and 1200 mg of chondroitin as base; after a dilution process, product samples were obtained at approximately the same concentration of prepared standards. Regarding the samples and standards reading in HPLC, initially each standard prepared was injected three times at the three concentration levels (80%, 100%, 120%), then each sample was injected only once, finally each standard (80%, 100%, 120% was injected again three times).

Analysis Method Validation

For analysis method validation, a batch corresponding to 300 sachets was used with product in the form of granules with an approximate weight of 3g ± 5%.

The raw materials necessary for the manufacture of a batch under the technique of wet granulation include the active ingredients in the form of glucosamine hydrochloride and chondroitin sulfate, also as excipients, colloidal silicon dioxide, polyvinylpyrrolidone, ethanol (removed by drying the granulate), a flavoring, in addition to sucralose and a coloring agent.

Being a product composed of two active ingredients, validation of the method of analysis for each active ingredient was carried out separately; therefore, two validations were carried out always using the same analysis method.

To determine the parameters to be evaluated it is necessary to know the category to which the test belongs to be validated, that is, if the method is official or non-official; In this case, being self-developed, it is a non-official method.

As can be seen in Table 1, the test is classified as category I, since it consists solely of a method of quantifying the active ingredients. Knowing this, Table 2 indicates the parameters to be evaluated in accordance with the Central American Technical Regulation (RTCA).

Table 1: Assays categories according to the type of analytical methodology.

Category	Analytical methodology
I	Methods for quantification of active ingredients in finished product (potency test, content uniformity).
II	Methods for the determination of impurities or degradation compounds in the finished product (related substances, degradation compounds).
III	Analytical methods for the determination of performance characteristics (dissolution, release of the drug).
IV	Identification tests (active ingredients).

Source: Central American Technical Regulation. Pharmaceutical products. Validation of analytical methods for the evaluation of the quality of medicines. (2006).

Table 2: Evaluation Parameters in a non-official analytical method validation.

Evaluation Parameters	Category I assay	Category II assay	Category III assay (quantitative)	Category III assay (Limit values)	Category IV assay
Accuracy	Yes	Yes	No	Yes	No
Precision	Yes	Yes	No	Yes	No
Specificity	Yes	Yes	Yes	Yes	Yes
Detection limit	No	No	Yes	No	No
Quantification limit	No	Yes	No	No	No
Linearity	Yes	Yes	No	Yes	No
Range	Yes	Yes	No	Yes	No
Repeatability	Yes	Yes	No	Yes	Yes

Source: Central American Technical Regulation. Pharmaceutical products. Validation of analytical methods for the evaluation of the quality of medicines. (2006).

Table 3: Work intervals according to analytical method.

Test	Interval
Active ingredient assay	80-120% of work concentration
Determination of impurities	50-120% of the specification
Content uniformity test	70-130% of work concentration
Dissolution test	± 20% of the specification

Source: ICH Harmonized Tripartite Guideline. Validation of analytical procedures: text and methodology Q2 (r1). (2005).

The work interval, as indicated in Table 3, for a Class I trial is 80-120%, therefore, this was the concentration of work used.

Parameter Evaluation

Specificity

For the evaluation of the specificity of the analytical method, this was the procedure:

1. Standards at 100% were prepared in triplicate; the same raw material employed in the manufacture of the batches of product duly certified by the laboratory was the standard.
2. A mass of 86.91 mg of placebo was weighed in triplicate and taken to a 100 mL graduated balloon; water was added to 80 mL and placed in an ultrasonic bath for 20 min. The balloon was allowed to cool and settled with distilled water.
3. The sample was then filtered through a 0.45um filter and taken to an HPLC vial.

Linearity

The procedure followed for the evaluation of this parameter to both the method and the system is as follows:

1. To determine the system linearity, standards were prepared in triplicate at 80, 90, 100, 110 and 120%.
2. To determine method linearity, samples were prepared at the same concentration levels as the standards prepared to evaluate the linearity of the system.
3. In the case of standards at 100%, the same prepared to evaluate the specificity were used.

Accuracy

Standards and samples of known concentration prepared for the determination of the linearity of the system were used at 80,100 and 120%.

Precision

The precision was determined with the recovery percentages obtained in the calculation of the accuracy made at 80,100 and 120%. Therefore, the same samples and standards used in the evaluation of the accuracy are used.

Repeatability

For the evaluation, three standards at 100% of those prepared above were used.

Subsequently, the analyst prepared six samples at 100% for both Glucosamine and Chondroitin as indicated by the method of analysis.

Finally, another analyst prepared six other samples in the same way for both active ingredients.

Verification of Parameters

Below are presented the results from the validation of the linearity parameter using the previously mentioned chondroitin standards.

System linearity

Table 4: Mass, concentration and areas of the chondroitin standards at different concentration levels for the evaluation of the linearity of the system, in the validation of the method of analysis for Chondroitin.

Concentration range (%) Chondroitin standards	Mass ± 0,01(g)	Concentration (ppm)	Area
80	17,98	287,7	1702,0
	17,59	281,4	1652,4
	17,87	285,9	1768,4
90	19,17	306,7	1905,5
	19,09	305,4	1910,4
	19,58	313,3	1960,0
100	22,48	359,7	2257,4
	21,69	347,0	2186,3
	22,28	356,5	2248,6
110	24,31	389,0	2424,8
	24,45	391,2	2471,1
	24,36	389,8	2468,9
120	27,17	434,7	2742,2
	26,46	423,4	2672,4
	26,34	421,4	2685,6

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Replicate one

Table 5: Concentration and areas of the chondroitin standards of replicate one, at different concentration levels for the evaluation of the linearity of the system, in the validation of the method of analysis for Chondroitin.

Concentration interval (%)	Concentration (ppm)	Reading of the areas
0	0,00	0,00
80	287,68	1702,00
90	306,72	1905,50
100	359,68	2257,40
110	388,96	2424,80
120	434,72	2742,20

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

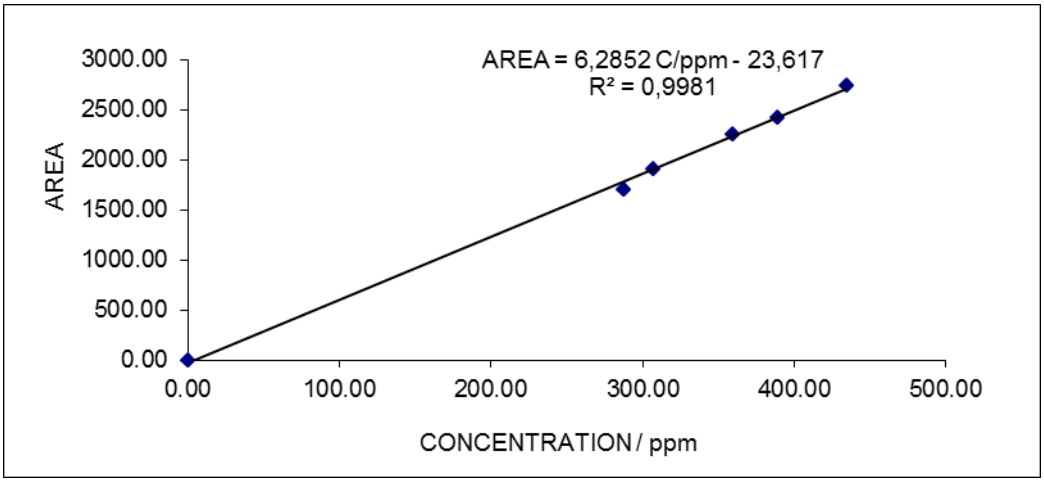

Figure 1: Linear regression of replicate one for determining the linearity of the system in the validation of the chondroitin analysis method.

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Table 6: Linear regression statistics for the evaluation of the linearity of the system in the replicate one. Validation of the method of analysis for Chondroitin.

Linear regression statistics		
Multiple correlation coefficient	0,99905858	
Determination coefficient R^2	0,99811804	
R^2 adjusted	0,99764755	
Typical error	47,219699	
Observations	6	
	Coefficients	*Typical error*
Intercept	-23,6172977	44,7925332
Slope	6,28521498	0,13645969

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Replicate two

Table 7: Concentration and chondroitin standards areas of replica two, at different concentration levels for the evaluation of the linearity of the system, in the validation of the method of analysis for Chondroitin.

Concentration interval (%)	Concentration (ppm)	Reading of the areas
0	0,00	0,00
80	281,44	1652,40
90	305,44	1910,40
100	347,04	2186,30
110	391,20	2471,10
120	423,36	2672,40

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

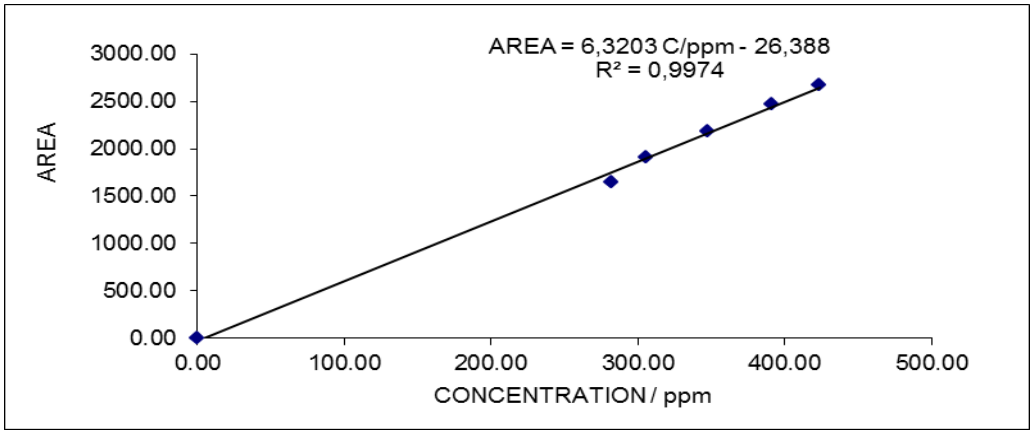

Figure 2: Linear regression of replicate two for determining the linearity of the system in the validation of the chondroitin analysis method.
Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Table 8: Linear regression statistics for the evaluation of the linearity of the system in the replicate two. Validation of the method of analysis for Chondroitin.

Linear regression statistics		
Multiple correlation coefficient	0,99867582	
Determination coefficient R^2	0,9973534	
R^2 adjusted	0,99669174	
Typical error	55,3676907	
Observations	6	
	Coefficients	*Typical error*
Intercept	-26,3880855	52,5491129
Slope	6,32030593	0,16279013

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Replicate three

Table 9: Concentration and chondroitin standards areas of replica three, at different concentration levels for the evaluation of the linearity of the system, in the validation of the method of analysis for Chondroitin.

Concentration interval (%)	**Concentration (ppm)**	**Reading of the areas**
0	0,00	0,00
80	285,92	1768,40
90	313,28	1960,00
100	356,48	2248,60
110	389,76	2468,90
120	421,44	2685,60

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

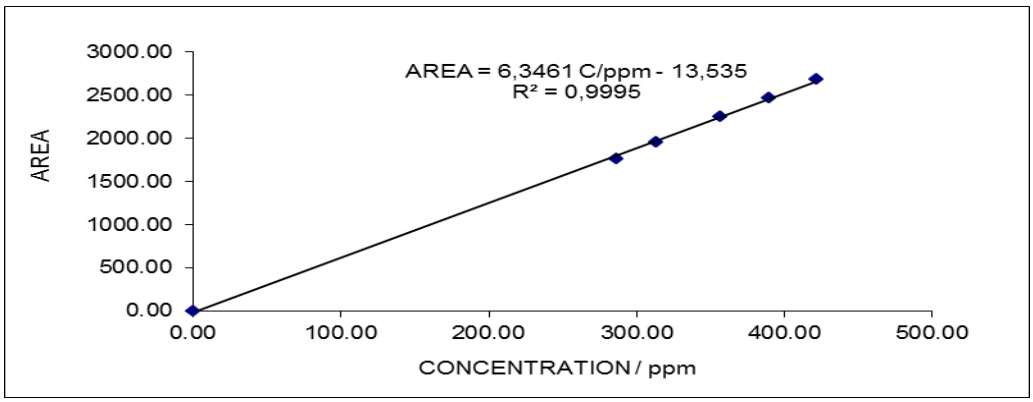

Figure 3: Linear regression of replicate three for determining the linearity of the system in the validation of the chondroitin analysis method.
Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Table 10: Linear regression statistics for the evaluation of the linearity of the system in the replicate three. Validation of the method of analysis for Chondroitin.

Linear regression statistics		
Multiple correlation coefficient	0,99977119	
Determination coefficient R^2	0,99954243	
R^2 adjusted	0,99942804	
Typical error	23,1408393	
Observations	6	
	Coefficients	*Typical error*
Intercept	-13,5352336	22,1118071
Slope	6,34605146	0,06788935

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

- Calculation of residuals

$$Practical\ Concentration = \frac{Area - Interception}{Slope}$$

$$Residual = Theoretical\ concentration - Practical\ concentración$$

$$Residual = 306,72 - 306,93 = -0,21$$

Calculation example
Replica 1 Sample at 90%

$$Practical\ concentration = \frac{1905,50 + 23,617}{6,2852} = 306,93$$

Table 11: Residues obtained for each of the Chondroitin standards at the five concentration levels established for each replica, in the validation of the linearity of the system for Chondroitin.

Replica	Concentration interval (%)	Reading	Theoretical concentration (ppm)	Practical concentration (ppm)	Residual
1	0	0,00	0,00	3,76	-3,76
	80	1702,00	287,68	274,55	13,13
	90	1905,50	306,72	306,93	-0,21
	100	2257,40	359,68	362,92	-3,24
	110	2424,80	388,96	389,55	-0,59
	120	2742,20	434,72	440,05	-5,33
2	0	0,00	0,00	4,18	-4,18
	80	1652,40	281,44	265,62	15,82
	90	1910,40	305,44	306,44	-1,00
	100	2186,30	347,04	350,09	-3,05
	110	2471,10	391,20	395,15	-3,95
	120	2672,40	423,36	427,00	-3,64
3	0	0,00	0,00	2,13	-2,13
	80	1768,40	285,92	280,79	5,13
	90	1960,00	313,28	310,99	2,29
	100	2248,60	356,48	356,46	0,02
	110	2468,90	389,76	391,18	-1,42
	120	2685,60	421,44	425,33	-3,89
				Summation	0,00

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

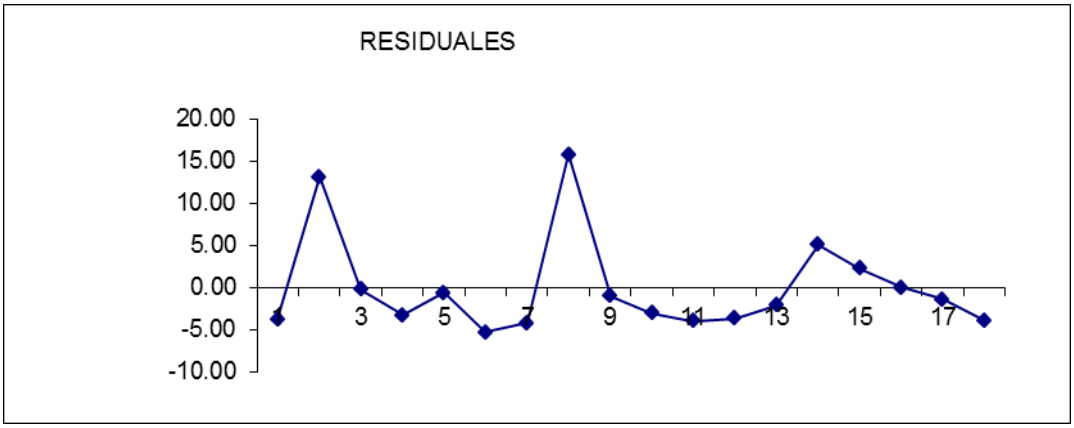

Figure 4: Residuals obtained in the determination of system linearity for the validation of the method of analysis of Chondroitin.

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

- Variance analysis

The variance analysis is performed for each replica by means of the t test for the slope, intercept and linear correlation coefficient as shown below:

Calculation example Replica 1:

Interception

$$t_{exp} = \frac{interception - 0}{s/\sqrt{n}}$$

$$t_{exp} = \frac{-23{,}6173 - 0}{44{,}7925/\sqrt{5}}$$

$$t_{exp} = 1{,}1790$$

$$t_{tab}\ (\alpha = 0{,}05) = 3{,}182$$

Where it is approved:

H_o= interception equals to 0; if texp < t_{tab}

H_1= interception is different from 0; if t_{exp} > t_{tab}

Being the t_{exp} < t_{tab} the null hypothesis (Ho) is approved, therefore it can be said that the intercept is equal to zero.

Slope

$$t_{exp} = \frac{slope - 0}{s/\sqrt{n}}$$

$$t_{exp} = \frac{6{,}2852 - 0}{0{,}1365/\sqrt{5}}$$

$$t_{exp} = 102{,}9607$$

$$t_{tab}\ (\alpha = 0{,}05) = 3{,}182$$

Where it is approved:

H_o= slope equals to 0; if texp < t_{tab}

H_1= slope is different from 0; if t_{exp} > t_{tab}

Being the t_{exp} > t_{tab} the null hypothesis (Ho) is rejected, therefore it can be said that the intercept is different from zero.

Linear correlation

$$t_{exp} = \frac{|r|\sqrt{n-2}}{\sqrt{(1-r^2)}}$$

$$t_{exp} = \frac{|0{,}9991|\sqrt{5-2}}{\sqrt{(1-0{,}9976)}}$$

$$t_{exp} = 35{,}3235$$

Where it is approved:

H_o= there is no linear correlation; if t_{exp} < t_{tab}

H_1= there is significant linear correlation; if t_{exp} > t_{tab}

Being the t_{exp} > t_{tab} the null hypothesis (Ho) is rejected, therefore it can be said that there is a significant linear correlation in the data.

For the other replicas, the same calculations were made and the following results were obtained.

Table 12: Results of the t-test of each of the three replicates for the validation of the system linearity for chondroitin.

Replicas	Interception T_{exp}	Slope T_{exp}	Linear correlation T_{exp}	T_{tab}	Result
1	1,1790	102,9607	35,3235	3,182	Intercept: It is equal to zero (H_o is accepted). Slope: Different from zero (H_1 is accepted). Linear correlation: There is linear correlation (H_1 is accepted).
2	1,1229	86,8097	30,1119	3,182	Intercept: It is equal to zero (H_o is accepted). Slope: Different from zero (H_1 is accepted). Linear correlation: There is linear correlation (H_1 is accepted).
3	1,3688	208,9884	70,6965	3,182	Intercept: It is equal to zero (H_o is accepted). Slope: Different from zero (H_1 is accepted). Linear correlation: There is linear correlation (H_1 is accepted).

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

- Criteria of acceptance

The r2 must be greater than 0.995 and less than 1.

The values of the residuals should not show trend.

Check that the slope is different from zero, the intercept is equal to zero and there is linear correlation through an analysis of variance.

- RESULT

As can be seen, in linear regression, for each of the replicas made to evaluate the linearity of the system, the acceptance criterion is reached, so it can be established that the linearity of the system is duly validated for the established work interval.

Therefore, working from 80 to 120%, the system is able to maintain linearity and ensures the accuracy of the data obtained.

In addition, it can be seen that the residues follow a random behavior, so that no significant trend is shown.

Finally, it is statistically shown that, by means of the test t analysis of variance, the slope for each line is different from zero, the intercept is equal to zero and there is a significant linear relationship between the data.

Linearity of the method

Table 13: Mass, concentration and areas of the product samples at different concentration levels, for the evaluation of the linearity of the method, in the validation of the method of analysis for Chondroitin.

Concentration interval (%) product samples	Mass (mg) ± 0,01	Concentration (ppm) with respect to chondroitin	Area
80	70,40	259,21	1684,5
	69,70	256,63	1702,0
	69,90	257,37	1690,7
90	78,10	287,56	1905,6
	79,30	291,98	1899,3
	78,90	290,51	1927,1
100	87,10	320,70	2096,1
	88,30	325,12	2103,4
	86,70	319,22	2101,7
110	95,90	353,10	2309,5
	95,60	351,99	2307,3
	95,40	351,26	2326,9
120	105,80	389,55	2512,7
	107,70	396,55	2565,6
	104,50	384,76	2548,0

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Replicate one

Table 14: Concentration and sample areas of replicate 1, at different concentration levels for the evaluation of the linearity of the method, in the Chondroitin method of analysis validation.

Concentration interval (%)	Concentration (ppm)	Reading of the areas
0	0,00	0,00
80	259,21	1684,50
90	287,56	1905,60
100	320,70	2096,10
110	353,10	2309,50
120	389,55	2512,70

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

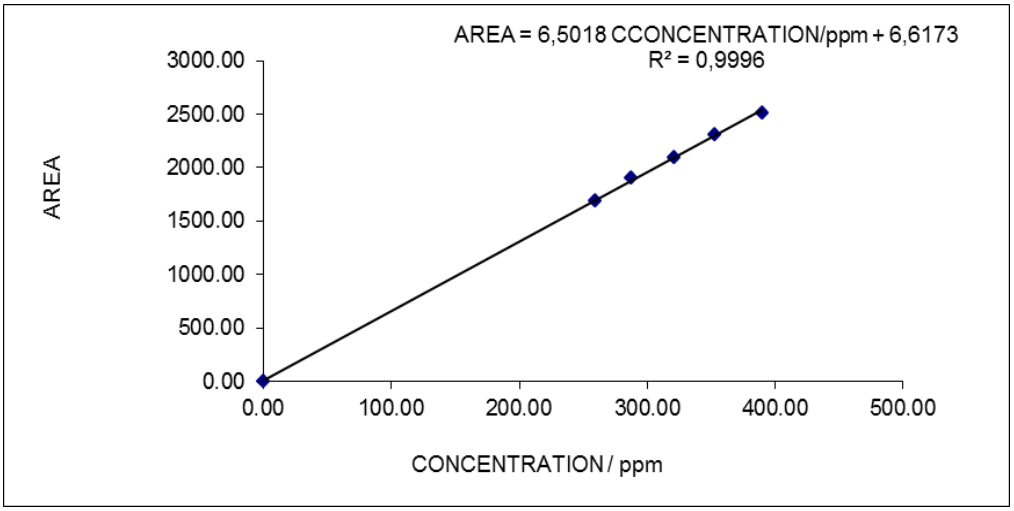

Figure 5: Linear regression of replicate 1 for the determination of the linearity of the method in the validation of the chondroitin analysis method.

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Table 15: Statistics of the linear regression for the evaluation of the linearity of the method in the replication 1. Validation of the method of analysis for Chondroitin.

Linear regression statistics		
Multiple correlation coefficient	0,999787915	
Determination coefficient R^2	0,999575875	
R^2 adjusted	0,999469844	
Typical error	20,8641801	
Observations	6	
	Coefficients	*Typical error*
Intercept	6,61732696	19,8865937
Slope	6,501839068	0,06696459

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Replicate two

Table 16: Concentration and sample areas of replicate 2, at different concentration levels for the evaluation of the linearity of the method, in the Chondroitin method of analysis validation.

Concentration interval (%)	**Concentration (ppm)**	**Reading of the areas**
0	0,00	0,00
80	256,63	1702,00
90	291,98	1899,30
100	325,12	2103,40
110	351,99	2307,30
120	396,55	2565,60

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

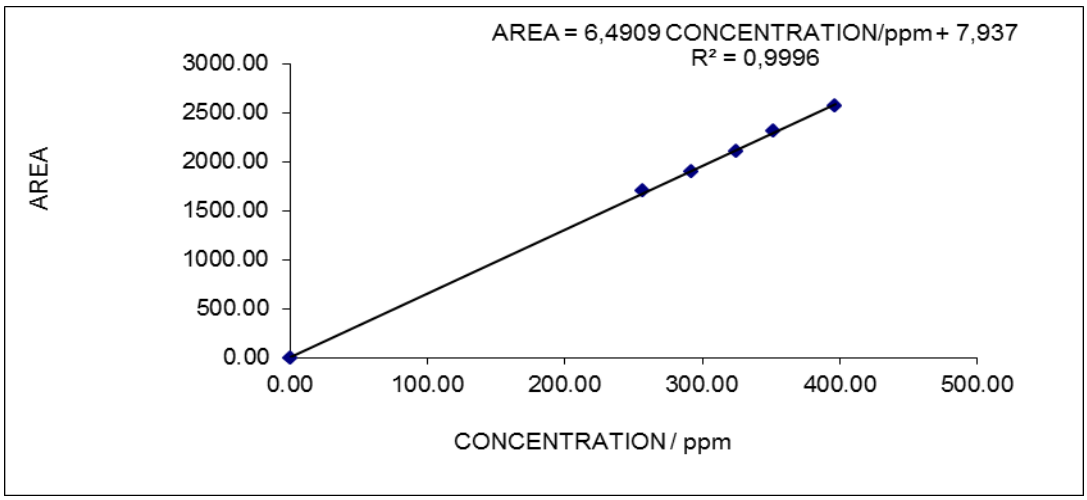

Figure 6: Linear regression of replicate 2 for the determination of the linearity of the method in the validation of the chondroitin analysis method.

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Table 17: Statistics of the linear regression for the evaluation of the linearity of the method in the replication 2. Validation of the method of analysis for Chondroitin.

Linear regression statistics		
Multiple correlation coefficient	0,99981173	
Determination coefficient R^2	0,99962349	
R^2 adjusted	0,99952937	
Typical error	19,8516435	
Observations	6	
	Coefficients	*Typical error*
Intercept	7,9369709	18,8600796
Slope	6,49091738	0,06298611

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Table 16: Concentration and sample areas of replicate 3, at different concentration levels for the evaluation of the linearity of the method, in the Chondroitin method of analysis validation.

Concentration interval (%)	Concentration (ppm)	Reading of the areas
0	0,00	0,00
80	257,368	1690,70
90	290,505	1927,10
100	319,224	2101,70
110	351,257	2326,90
120	384,763	2548,00

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

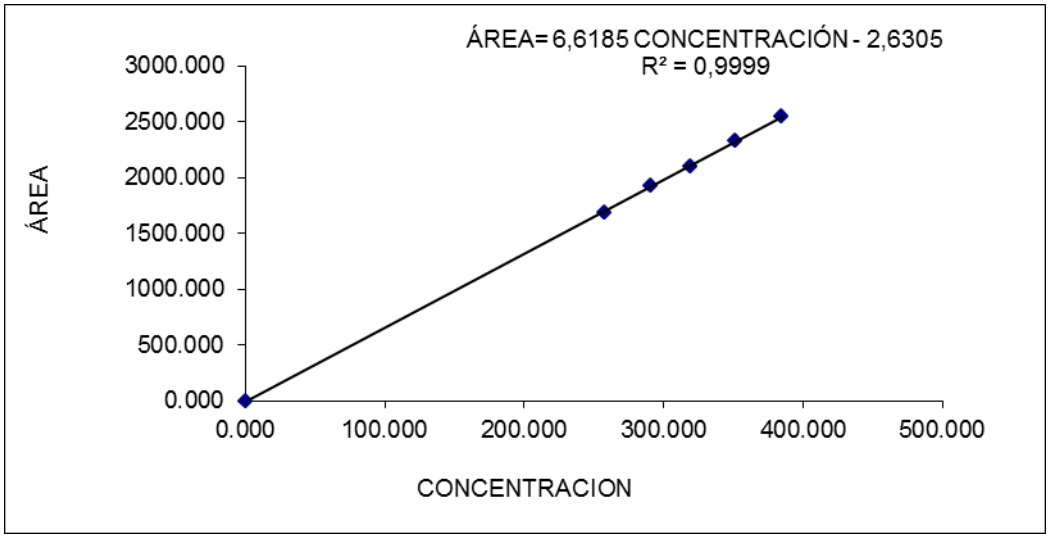

Figure 7: Linear regression of replicate 3 for the determination of the linearity of the method in the validation of the chondroitin analysis method.
Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

Table 19: Statistics of the linear regression for the evaluation of the linearity of the method in the replication 3. Validation of the method of analysis for Chondroitin.

Linear regression statistics		
Multiple correlation coefficient	0,99996802	
Determination coefficient R^2	0,99993604	
R^2 adjusted	0,99992006	
Typical error	8,18419246	
Observations	6	
	Coefficients	*Typical error*
Intercept	-2,63052819	7,82086408
Slope	6,61846931	0,02646558

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

- Calculation of residuals

$$Practical\ Concentration\ = \frac{Area - Interception}{Slope}$$

$$Residual = Theoretical\ concentration - Practical\ concentración$$

Calculation example:
Replica 1 Sample at 80%

$$Practical\ concentration = \frac{1684.5 - 6.6173}{6,5018} = 258.06$$

$$Residual = 259.21 - 258.06 = 1.15$$

Table 20: Residues obtained for each of the Chondroitin standards at the five concentration levels established for each replica, in the validation of the linearity of the method for Chondroitin.

Replica	Concentration interval (%)	Reading	Theoretical concentration (ppm)	Practical concentration (ppm)	Residual
1	0	0,00	0,00	-1,02	1,02
	80	1684,50	259,21	258,06	1,15
	90	1905,60	287,56	292,07	-4,51
	100	2096,10	320,70	321,37	-0,67
	110	2309,50	353,10	354,19	-1,09
	120	2512,70	389,55	385,44	4,11
2	0	0,00	0,00	-1,22	1,22
	80	1702,00	256,63	260,99	-4,36
	90	1899,30	291,98	291,39	0,59
	100	2103,40	325,12	322,83	2,29
	110	2307,30	351,99	354,24	-2,25
	120	2565,60	396,55	394,04	2,51
3	0	0,00	0,00	0,40	-0,40
	80	1690,70	257,37	255,85	1,52
	90	1927,10	290,51	291,57	-1,06
	100	2101,70	319,22	317,95	1,28
	110	2326,90	351,26	351,97	-0,72
	120	2548,00	384,76	385,38	-0,62
				Summation	0,00

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

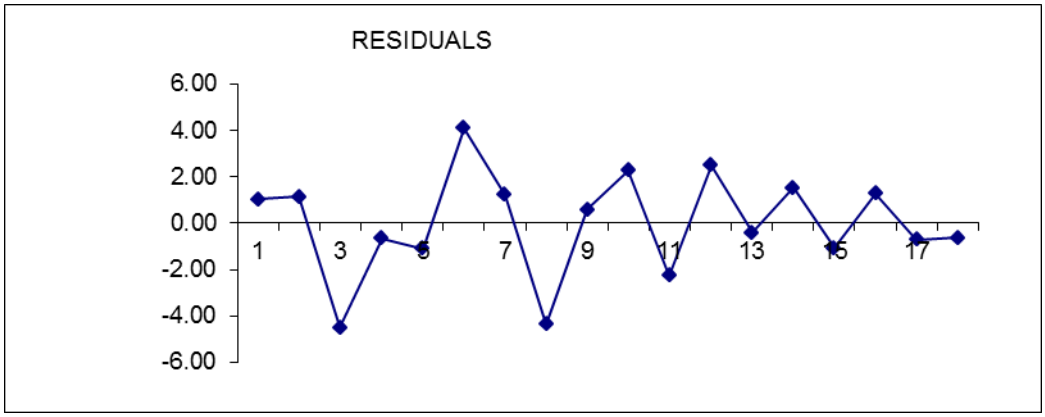

Figure 8: Residuals obtained in the determination of method linearity for the validation of the method of analysis of Chondroitin.
Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

- Variance analysis

The variance analysis is performed for each replica by means of the t test for the slope, intercept and linear correlation coefficient as shown below:

Calculation example Replica 1:

Interception

$$t_{exp} = \frac{interception - 0}{s/\sqrt{n}}$$

$$t_{exp} = \frac{6.6173 - 0}{19.8866/\sqrt{5}}$$

$$t_{exp} = 0.7441$$

$$t_{tab}\ (\alpha = 0{,}05) = 3{,}182$$

Where it is approved:

H_o= interception equals to 0; if texp < t_{tab}

H_1= interception is different from 0; if $t_{exp} > t_{tab}$

Being the $t_{exp} < t_{tab}$ the null hypothesis (Ho) is approved, therefore it can be said that the intercept is equal to zero.

Slope

$$t_{exp} = \frac{slope - 0}{s/\sqrt{n}}$$

$$t_{exp} = \frac{6{,}5018 - 0}{0{,}0670/\sqrt{5}}$$

$$t_{exp} = 216.9920$$

$$t_{tab}\ (\alpha = 0{,}05) = 3{,}182$$

Where it is approved:

H_o= slope equals to 0; if texp < t_{tab}

H_1= slope is different from 0; if $t_{exp} > t_{tab}$

Being the $t_{exp} > t_{tab}$ the null hypothesis (Ho) is rejected, therefore it can be said that the intercept is different from zero.

Linear correlation

$$t_{exp} = \frac{|r|\sqrt{n-2}}{\sqrt{(1-r^2)}}$$

$$t_{exp} = \frac{|0{,}9998|\sqrt{5-2}}{\sqrt{(1-0{,}9995)}}$$

$$t_{exp} = 77.4442$$

Where it is approved:

H_o= there is no linear correlation; if $t_{exp} < t_{tab}$

H_1= there is significant linear correlation; if $t_{exp} > t_{tab}$

Being the $t_{exp} > t_{tab}$ the null hypothesis (Ho) is rejected, therefore it can be said that there is a significant linear correlation in the data.

For the other replicas, the same calculations were made and the following results were obtained.

Table 21: Results of the t-test of each of the three replicates for the validation of the method linearity for chondroitin.

Replicas	Interception T_{exp}	Slope T_{exp}	Linear correlation T_{exp}	T_{tab}	Result
1	0,7441	216,9920	77,4442	3,182	Intercept: It is equal to zero (H_o is accepted). Slope: Different from zero (H_1 is accepted). Linear correlation: There is linear correlation (H_1 is accepted).
2	0,9410	230,6735	77,4442	3,182	Intercept: It is equal to zero (H_o is accepted). Slope: Different from zero (H_1 is accepted). Linear correlation: There is linear correlation (H_1 is accepted).
3	0,7521	558,4685	173,2051	3,182	Intercept: It is equal to zero (H_o is accepted). Slope: Different from zero (H_1 is accepted). Linear correlation: There is linear correlation (H_1 is accepted).

Source: Lester Rojas Quesada. Laboratorios Stein Costa Rica. (2014).

- Criteria of acceptance

The r2 must be greater than 0.995 and less than 1.

The values of the residuals should not show trend.

Check that the slope is different from zero, the intercept is equal to zero and there is linear correlation through an analysis of variance.

- RESULT

As in the validation of the linearity of the system, it can be observed that the linear regression for each one of the replicas made to evaluate the linearity of the method fulfills the acceptance criterion, so it can be established that the linearity of the method is duly validated for the established work interval.

It can also be said that, working from 80 to 120% concentration, the method is able to maintain linearity and ensures the accuracy of the data obtained.

In addition, it can be seen that the residues follow a random behavior, so that no significant trend is shown.

Finally, it is statistically shown that, by means of the test t analysis of variance, the slope for each line is different from zero, the intercept is equal to zero and there is a significant linear relationship between the data.

REFERENCES

1. Abad Santos, F., Ochoa Mazarro, D., & García García, A. (2011). Actualización de la eficacia de condroitín sufato y sulfato de glucosamina en el tratamiento de artrosis. Actualidad en Farmacología y Terapéutica, 9: 97-108.
2. International Conference of Harmonization. (2005). *Validation of analytical procesures: Text and methodology* . Harmonised Tripartite Guideline.
3. Reglamento Técnico Centroamericano. (2006). *Productos farmacéuticos: Validación de métodos analíticos para la evaluación de la calidad de los medicamentos.*
4. Rojas Quesada, L. (2015). Diseño y desarrollo de un producto farmacéutico multiorigen en combinación de glucosamina y condroitina en todas las fases previas al registro sanitario para su aprobación y posterior comercialización en el mercado farmacéutico. San José, Costa Rica. Universidad Latina de Costa Rica.
5. Sweetman, S. C. (2009). Martindale: The Complete Drug Reference. Grayslake, USA: Pharmaceutical Press.