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Research Paper

Simultaneous Estimation of Glucosamine Sulfate and Chondroitin Sulfate Mixture

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Abstract: *A simple, reliable high-performance liquid chromatographic (HPLC) method has been developed and validated for the simultaneous estimation of glucosamine Sulfate KCl and chondroitin sulfate mixture. Both drugs were separated on a C18 column (250 mm×4.6mm×5µm) using mobile phase that was 99.5:0.5 (v/v) mixture of potassium phosphate buffer of pH 3.0 (orthophosphoric acid and potassium hydroxide) and acetonitrile respectively, pumped at a flow rate of 1.0 ml min^{-1}, using RI detector at 25°C. The method was first optimized for mobile phase, temperature, flow rate and then validated according to ICH guidelines. The validation characteristics included accuracy, precision, linearity, range, specificity and limit of quantitation. Robustness testing was also conducted to evaluate the effect of minor changes to the chromatographic system and to establish appropriate system suitability parameters. The method showed adequate separation of glucosamine sulfate KCl and chondroitin sulfate sodium from its stress induced degradation products. Validation acceptance criteria were met in all cases. The applicability of the method was demonstrated by determining the drug content of two commercial pharmaceutical formulations, where it exhibited good performance.*

Keywords: HPLC validated method, Glucosamine Sulfate KCl, chondroitin sulfate sodium .

Introduction

Glucosamine an amino monosaccharide is found in chitin, glycoproteins and in GAGSs. Glucosamine is the main building block of amino sugar and an important fundamental constituent of interestial proteins and cell wall [1]. Glucosamine is a hexosamine sugar that is six carbon amino sugars and commonly found in human cells. Glucosamine is a modified glucose by structure, that replaces –OH group with an $–NH_3$ group present on carbon two (C-2)[1].

Glucosamine is commercially available as a nutritional supplement in three forms *i.e.* glucosamine KCl or HCl, glucosamine sulfate and N-acetyl-glucosamine. All these forms are water soluble and glucosamine salt act as a delivery vehicle. They absorbed in small intestine and then transported to the body via portal circulation, to the liver. It appears that a significant fraction of the ingested glucosamine is catabolized by first-pass metabolism in the liver [2]. Glucosamine is preferentially incorporated by chondrocytes into the components of the GAG chains in intact cartilage and stimulates the synthesis of physiological proteolglycans. It also decreases the activity of catabolic enzymes [3-5].

In certain tissues, glucosamine has a higher affinity for glucose transporters than glucose itself and is incorporated into glycoproteins faster than glucose[6]. It also inhibits the degradation of articular cartilage induced[7]**.** This supports the suggestion that exogenous glucosamine acts mainly as a substrate for biosynthesis of muco polysaccharides and biopolymers of joints and bones and, thus, contributes to restoration of damaged cartilage[2,8]**.** Chondroitin naturally occurs in human cartilage, bone, skin, cornea, and arterial walls. It is present in the form of sulfates or with sulphuric acid. Its natural origin is shark or bovine cartilage. It can also be prepared artificially in lab. It is the most abundant glycosaminoglycan (long chain of specialized polysaccharides) in human cartilage and keeps it elastic. Chondroitin sulfate's presence is essential for the

production of cartilage. It produces Proteoglycan; a fundamental and imperative component of cartilage tissue. Basically chondroitin sulfate holds water for the proteoglycan, which is vital for the proper functioning of joints. Healthy joints without chondroitin sulfate cannot be imagined. Structurally, Chondroitin sulfate consists of alternating disaccharide units of glucuronic acid (simple ringed carboxylic acid, 6^{th} carbon oxidized to carboxylic acid, $C_6H_{10}O_7$) and galactosamine (alpha-D-glucosamine) attached to protein to form a part of proteoglycan [9]**.** It is categorized as symptomatic slow-acting drug due to its slow onset effect on cartilage recovery and anti-inflammatory activity. Chondroitins show first order kinetics up to a dose of 30,000mg [10-13]. Chondroitin plays a vital role in the production, growth, and metabolism of glycosaminoglycan. It also activates the formation of proteoglycans in human cartilage tissues. Its presence is very important for the protection of collagen as it not only slows the breakdown of collagen but also enhances its activity. Chondroitin also holds inflammation [14], it inhibits the production of the stromelysin which is responsible for the degradation of the cartilage tissue [15]**.** For the protection of chondrocytes, chondroitin suppress the synthesis of nitric acid [16].

The originator of the glycosaminoglycan, which is used to repair bone cartilage, is glucosamine and the most profuse glycosaminoglycan is chondroitin. The combination of the two drugs drawn interest due to the relation mentioned above. In combination both help to counteract narrowing of joint space, loss of cartilage, and enhance the production of cartilage. It is observed that glucosamine help out in the production of cartilage while chondroitin inhibits or tends to inhibit the destruction of cartilage. This combination also helps in the relief of pain. In contrast to NSAIDs, the both in combination have almost no side effects.

Glucosamine sulfate KCl and Chondroitin sulfate sodium are very effective in the treatment of the disease 'osteoarthritis', a type of arthritis; a degenerative disease of the joints of bone. In severe cases inflammation occurs and finally immobility of joints. There is a method available for glucosamine sulfate KCl estimation, but not much précised or validated whereas there is no combined method for glucosamine sulfate KCl and chondroitin sulfate sodium available. Furthermore the method was developed using RI detector to obtain the précised and accurate results.

Material and Methods

Glucosamine sulfate KCl and Chondroitin sulfate sodium samples were obtained from Schazoo Pharmaceuticals. Sodium hydroxide, Hydrochloric acid, Orthophosphoric acid, and Hydrogen peroxide were purchased from Merck. Acetonitrile, Methanol and Double distilled water were of HPLC grade.

Preparation of mobile phase and samples

Mobile phase was prepared by dissolving acetonitrile in phosphate buffer in 0.5:99.5 ratios. Phosphate buffer was prepared by dissolving 2ml of orthophosphoric acid in 2000ml of distilled water. pH of the buffer was maintained at pH 3.0. Samples were soluble in water so there solutions were made in it. For the performance of degradation studies 1.5M HCl solution for acidic degradation, 1.00M NaOH solution for basic degradation, 30% H_2O_2 for oxidative degradation were prepared.

Instrumentation

HPLC ECOM manual machine was used for analysis of the samples. The system was equipped with the column oven, pump, injector, and RI detector.

Chromatographic conditions

The column used was C18 4.6× 250mm, 5µm Merck. The mobile phase was 99.5:0.5 phosphate buffer and acetonitrile respectively. Flow rate was set on 1ml/min. Column temperature was maintained on 28°C. Detector used was RI detector. Total run time was 7 minutes.

Results and Discussion

Method development

The endeavor for developing a method for glucosamine and chondroitin sulfate was to get a simple, instant, precise, accurate, and above all simultaneous method for both samples in their fixed dosage forms. Initially the work was started with testing different mobile and stationary phases. The method was advanced on HPLC by taking mobile phase made up of combination of phosphate buffer (pH 3.0) and acetonitrile in different v/v ratios trying available columns (stationary phase) such as C8, CN, and C18. Many tribulations were faced while taking trails, for example, broadening of peaks, tailing factor, and improper separation. Every time a single change was brought while keeping all other parameters and conditions constant. At last C18 column was decided as it bestowed good quality separation, comparatively to others. After finalizing Stationary phase I switched on to the selection of mobile phase by varying its pH first. Trials were taken for different pH ranging from 2.5 to 3.5. pH 3.0 was decided to carry the method with due to its optimized results so far. The ratio of 99.5:0.5 of phosphate buffer and acetonitrile, respectively, generated the most suitable and well separated peaks so it was finalized. At beginning all this work was being performed on UV detector at 195nm wave length. With UV detector we were unable to get proper separation of peaks. Beside this, several small peaks were also observed every time.

In addition to this 195nm range was also not suitable as it is far UV range, more towards visible range. Thus RI detector was taken in consideration and decided to have trails on it. As RI is a temperature sensitive technique, consequently column was also kept in column oven. At last at 28°C temperature, with C18 column and 99.5:0.5 phosphate buffer:acetonitrile mobile phase at the flow rate of 1ml/min the most optimized and well separated peaks were observed. Separated peaks of Glucosamine sulfate KCl and Chondroitin sulfate sodium can be obtained at retention times of 1.8±0.02 minutes and 3.00±0.03 minutes respectively. Representative chromatogram of Glucosamine sulfate KCl and Chondroitin sulfate sodium are shown in Figure 1. The chromatogram shows simultaneous analysis of both drugs.

The proposed stability indicating HPLC method was validated according to ICH guidelines [17].

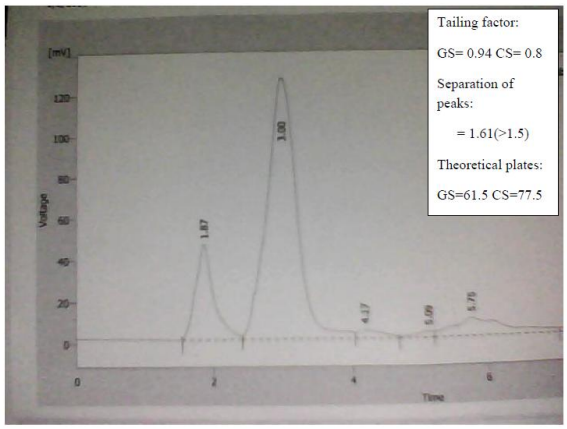

Figure 1: Chromatogram of Chondroitin Sulfate Sodium at 1.8 min and Glucosamine Sulfate KCl at 3.00 min

Method validation

Linearity

Linearity of the developed method was performed by analyzing six concentrations ranging from 20mg/ml to 80mg/ml that was 20mg/ml, 30mg/ml, 40mg/ml, 50mg/ml, 60mg/ml, and 80mg/ml. For each dilution triplicate samples were prepared and then analyzed. Linear regression equation was resolved by taking peak area against the concentration of the analyte and correlation coefficient was calculated. The linear regression equation for Glucosamine was calculated y=1421x-297.0, and for chondroitin it was y=383x-6.266. The correlation coefficient was 0.996 for glucosamine and 0.975 for chondroitin. The linearity graph is shown in figures 2 and 3.

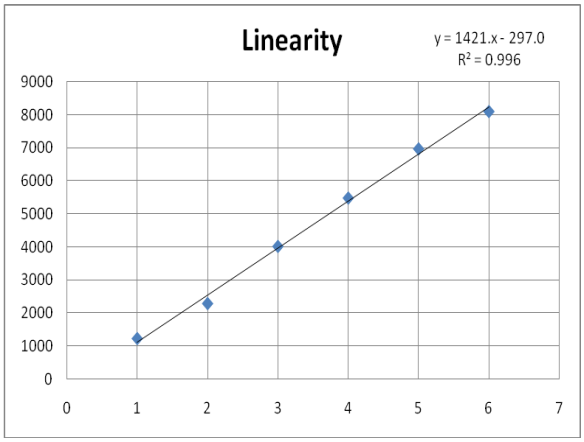

Figure 2: Linearity Graph for glucosamine sulfate KCl

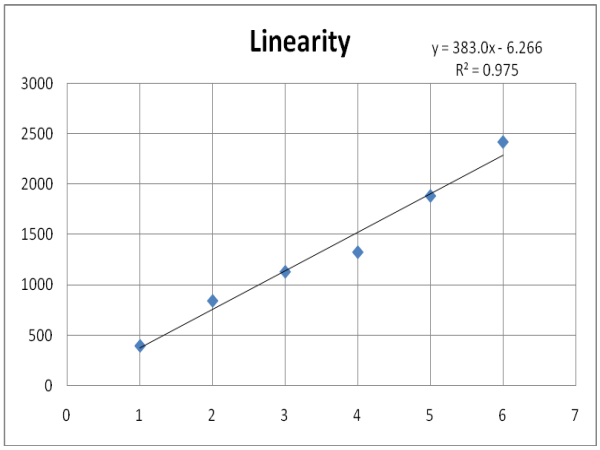

Figure 3: Linearity Graph for chondroitin sulfate sodium

Limit of detection and quantitation

Limit of detection and quantitation were calculated on the basis of standard deviation of response and slope. The calculations for LOD for glucosamine and chondroitin were 0.31µg/ml and 1.65µg/ml, and LOQ readings were 1.105µg/ml and 5.00µg/ml, respectively.

Accuracy

The evaluation of accuracy was done with taking the mean recovery of three replicate peaks area for each level as mentioned in experiment. Calculations of recoveries were performed by comparing spiked with the peak area of blank samples. Recoveries were calculated in percent recoveries. It ranged from 50.94% to 67.41% for glucosamine sulfate KCl, and 76.05% to 87.13% for chondroitin sulfate. It is shown in table 1.

Table 1: Recovery experiments for calculating accuracy of glucosamine sulfate KCl and chondroitin sulfate

Drugs	Concentration added mg/ml	Concentration recovered mg/ml (Mean)	Accuracy (%)
Glucosamine Sulfate KCl	40 mg/ml	20.44 mg/ml	51.11%
	50 mg/ml	27.32 mg/ml	54.64%
	60 mg/ml	40.44 mg/ml	67.41%
Chondroitin sulfate Sodium	40 mg/ml	30.42 mg/ml	76.05%
	50 mg/ml	43.65 mg/ml	87.13%
	60 mg/ml	50.42 mg/ml	84.03%

Precision

The intra and inter day precision was calculated for three different concentrations for recovery study. The intraday precision was calculated for three replicates of each concentration ranged from 20mg/ml to 80mg/ml. The inter day precision was calculated for the same samples for the three consecutive days. The results are given in table. The stability was observed by keeping them at room temperature for three days and at 10°C for a week.

Table 2: Precision of proposed HPLC method for glucosamine sulfate KCl and chondroitin sulfate sadium

Drug	Concentration taken mg/ml	Concentration Recovered mg/ml	Accuracy %	Precision %RSD
Glucosamin Sulfate KCL	20mg/ml	9.81 mg/ml	49.05%	0.064%
	30mg/ml	18.2 mg/ml	60.07%	0.459%
	40mg/ml	31.9 mg/ml	79.81%	0.240%
	50mg/ml	43.5 mg/ml	87.07%	0.111%
	60mg/ml	55.3 mg/ml	92.23%	0.137%
	80mg/ml	64.3 mg/ml	80.38%	0.107%
Chondroitin Sulfate Sodium	20mg/ml	9.88 mg/ml	49.40%	2.086%
	30mg/ml	20.9 mg/ml	69.76%	1.239%
	40mg/ml	28.0 mg/ml	70.13%	0.536%
	50mg/ml	32.8 mg/ml	65.72%	0.131%
	60mg/ml	46.6 mg/ml	77.75%	0.371%
	80mg/ml	59.8 mg/ml	74.85%	0.218%

Table 3: Between day Precision of proposed HPLC method for glucosamine sulfate KCl and chondroitin sulfate sadium

Drug	Concentration taken mg/ml	Concentration Recovered mg/ml	Accuracy %	Precision %RSD
Glucosamin Sulfate KCL	20mg/ml	9.69 mg/ml	34.97%	0.782%
	30mg/ml	17.9 mg/ml	59.99%	0.291%
	40mg/ml	31.5 mg/ml	78.88%	0.206%
	50mg/ml	43.0 mg/ml	86.06%	0.178%
	60mg/ml	54.6 mg/ml	91.15%	0.138%
	80mg/ml	63.5 mg/ml	79.45%	0.100%
Chondroitin Sulfate Sodium	20mg/ml	11.6 mg/ml	58.35%	0.891%
	30mg/ml	24.1 mg/ml	80.46%	0.520%
	40mg/ml	31.9 mg/ml	79.87%	0.413%
	50mg/ml	40.1 mg/ml	80.22%	0.143%
	60mg/ml	54.4 mg/ml	90.76%	0.140%
	80mg/ml	72.1 mg/ml	90.16%	0.144%

Specificity of method

Degradation studies were carried out to determine the specificity of the method. Glucosamine sulfate and chondroitin sulfate were subjected to acidic, alkaline, and oxidative stress for degradation. To carry out acid degradation 1ml of the working sample solution was taken in a flask, 1ml of 1.5M HCl solution was poured in it and made volume up to 10ml and placed for two hours. For basic degradation 1ml of the sample solution was mixed with 1ml of 1M NaOH and made volume up to 10ml with dist. water. It was also left for two hours. For oxidative degradation 1ml of H_2O_2 30% was taken and mixed with 1ml of sample solution, made volume up to 10ml and placed for two hours. All the three were prepared in triplicates and then injected for the analysis. The results were summarized in Table 4.

Table 4: Degradation studies of glucosamine sulfate KCl and chondroitin sulfate sodium

Drugs	Nature of stress	N	% Degradation	% Recovery
Glucosamine Sulfate KCl	HCl 1.5M	3	2.416%	97.58%
	NaOH 1M	3	8.417%	91.54%
	H_2O_2 30%	3	10.89%	89.11%
Chondroitin Sulfate Sodium	HCl 1.5M	3	20.84%	79.15%
	NaOH 1M	3	27.7%	72.3%
	H_2O_2	3	35.69%	64.31%

Robustness

Minor intentional variations were made to modify chromatographic conditions for the determination of robustness of proposed method. Chromatographic conditions like pH and composition of mobile phase and flow rate were changed slightly.

Under such modified conditions the chromatographic parameters such as retention time, theoretical plates, and tailing factor were calculated and studied against the optimized conditions. It was observed that minor chromatographic change does not change the results too much. The observations and calculations are given in table 5.

Table 5: Robustness studies of glucosamine sulfate KCl and chondroitin sulfate sodium

Chromatographic Conditions	Retention time	Tailing factor	Theoretical plate
Acetonitrile: Buffer 5:95	2.63 min for CS 4.02 min for GS	1.14 0.84	31.66 110.5
Acetonitrile: Buffer 3:97	2.69 min for CS 4.05 min for GS	1.28 0.81	27.83 75.09
Flow rate 0.5ml/min	2.14 min for CS 3.65 min for GS	1.16 0.92	70.47 43.67
Flow rate 1.5ml/min	1.79 min for CS 2.93 min for GS	1 0.95	71.00 58.71
Buffer pH 2.5	Complex peak for CS 4.14 min for GS	-- 0.83	--- 78.47
Buffer pH 3.5	2.23 min for CS 3.73 min for GS	1.32 0.63	56.22 63.70

Conclusion

The method developed is simple, rapid, and selective method for the simultaneous estimation of glucosamine sulfate KCl and chondroitin sulfate sodium. As there was no simultaneous method for glucosamine sulfate KCl and chondroitin sulfate sodium, so the developed method can provide the analysis of both in just seven minutes with quite fair separation. Further the previous methods were not validated and provided by vendors while this method is a validated method and can be adopted for the analysis of both drugs in pharmaceutical formulations.

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