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Colorimetric estimation of Chondroitin Sulfate in bulk drug and pharmaceutical formulation using cationic dye Methylene Blue

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ABSTRACT

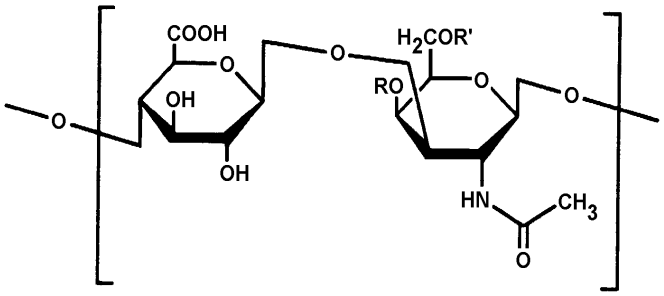
A highly sensitive and selective colorimetric method was developed for the determination of chondroitin sulfate in bulk drug and tablet dosage form. The proposed colorimetric method has been applied to quantify chondroitin sulfate by interacting with methylene blue. It is based on the principle of decrease in the absorbance of methylene blue at 664 nm on complexation with chondroitin sulfate. The Beer-Lambert's law obeyed in the range of 0.5 - 4 µg/ml with correlation coefficient at 0.9985. The results presented are statistically validated in accordance with the guidelines provided by ICH. The recovery studies were carried out at three different levels. The precision was good with RSD lower than 2.0 %. The developed method was found to be considerably easy, simple, specific, precise, reproducible and cost effective.

Keywords: spectrophotometric method, chondroitin sulfate, methylene blue.

INTRODUCTION

Chondroitin sulfate (CHS) is a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars galactosamine and glucuronic acid. It is usually found attached to proteins as part of a proteoglycan**.** GAGs are large complexes of negatively charged heteropolysaccharides chains generally associated with small amount of protein, which are formerly known as mucopolysaccharides [1,2]. GAGs in the form of proteoglycans comprise the ground substance in the extra cellular matrix of connective tissue. CHS is chemically Poly- (1- 3)-N-acetyl-2-amino-2-deoxy-3-O-ß-D-glucopyranurosyl – 4 - (or 6-) sulfonyl-D-galactose. The amino group of galactosamines in the basic unit of CHS is acetylated, yielding N-acetyl-galactosamine. The sulfate group is esterified to the carbon 4 or 6 position in N-acetyl-galactosamine yield Chondroitin sulfate A and Chondroitin sulfate C respectively.

The molecular weight of chondroitin sulfate A or C ranges from 5,000 to 50,000 Daltons and contains about 15 to 150 basic units of D-galactosamine and D-glucuronic acid[3]. It is represented by the following structural formula:



Chondroitin sulfate A R = SO_3H R' = H
Chondroitin sulfate C R = H R' = SO_3H

Figure 1- Chemical structure of Chondroitin sulfate

Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression. Chondroitin sulfate mainly used in promotion and maintenance of the structure and function of cartilage, pain relief of osteoarthritic joints and anti-inflammatory activity. It has been known for chondroitin sulfate can bring significant pain relief and enhanced mobility in osteoarthritis. chondroitin sulfate has the ability to bind to receptor sites on synovial cell surfaces and thus induce production of hyaluronic acid, crucial to joint mobility.

Literature review revealed that few analytical methods include gel-exclusion chromatography[4,5], capillary electrophoresis[6] and titration with cetyl pyridinium chloride detecting end point with a phototrode[7,8]. The other methods in the literature for quantitating chondroitin sulfate needs enzymatic digestion followed by disaccharide analyses, include reverse phase high performance liquid chromatography[9] (HPLC), anion-exchange chromatography[10] and gel electrophoreses with fluorescence detection[11].

Hence an attempt has been made to develop accurate, precise and reproducible colorimetric method for estimation of chondroitin sulfate in bulk drug and tablet formulation. The method was validated by using various parameters as per ICH guidelines [12].

MATERIALS AND METHODS

Instrumentation

Perkin Elmer double beam UV-Visible spectrophotometer was used with 1 cm matched quartz cells.

Chemicals and Reagents

Pure chondroitin sulfate was obtained as gift sample from Banner Pharmacaps (India) Pvt Ltd, Bangalore, India, carbazole (Rolex AR Grade), methylene blue (Qualigens fine chemicals) and demineralised water were used.

Reagent:

Methylene blue solution: 15 mg of methylene blue was dissolved in about 75 ml of demineralised water in 100 ml volumetric flask and sonicated for 5 minute. Shaken well,

volume was made up with demineralised water to 100ml. volume of 25 ml of above solution was diluted to 100 ml with demineralised water to get a concentration of 37.5µg/ml

Preparation of Standard Stock Solution

About 100 mg of chondroitin sulfate working reference standard of known purity (92.42% pure) was accurately weighed into a 100 ml volumetric flask, dissolved & volume was made up to 100 ml with demineralised water (1mg/ml). Volume of 5 ml of stock solution was further diluted to 100 ml with demineralised water to get a concentration of 50µg/ml.

Determination of Wavelength of Maximum Absorbance of Methylene Blue

10 ml of methylene blue solution was pipetted into 50 ml volumetric flask and the volume was made up with demineralised water. The volumetric flask was shaken well for 30 minutes by inversion for about 5 minutes intervals. The solution was scanned in the spectrum mode over the range of 400-800nm against water as a blank. The wavelength of maximum absorbance of methylene blue was found at 664 nm as shown in figure no. 2

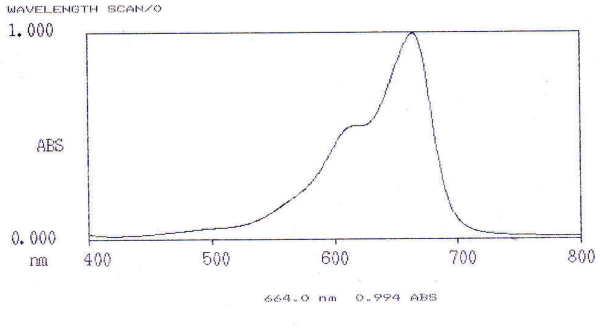


Figure 2 - Absorbance maxima of methylene blue

Complex formation of Chondroitin sulfate with Methylene blue:

Methylene blue is a cationic dye that interacts with chondroitin sulfate and decreases in absorbance of methylene blue at λ_{max} 664 nm. The decrease in the absorption at 664 nm is proportional to the concentration of chondroitin sulfate in the solution, providing a basis for the quantitative determination of chondroitin sulfate by methylene blue.

With the addition of increasing concentration of chondroitin sulfate, there is decrease in absorbance of methylene blue at 664 nm**.** The change in absorbance indicates that chondroitin sulfate has interacted with methylene blue molecule. Because there were two negative charges in each repeating disaccharide unit of chondroitin sulfate and cationic dye methylene blue, electrostatic force might be involved in the interaction as driving force.

The dye molecules were binding with chondroitin sulfate by an electrostatic force at the first stage and then undergo polymerization due to hydrophobic bonds or other dye-dye bonds. This is shown by stacking model for chondroitin sulfate - methylene blue complex [13] as shown figure no 3.

Spectrophotometric titrations were carried out by reading the absorbance at 664 nm by a series of methylene blue solutions of fixed volume containing fixed amount of methylene blue and increasing amount of the chondroitin sulfate. The absorbance was measured relative to water. The actual absorbance value was obtained by the absorbance of methylene solution subtracted by the respective absorbance of chondroitin sulfate in methylene blue solution.

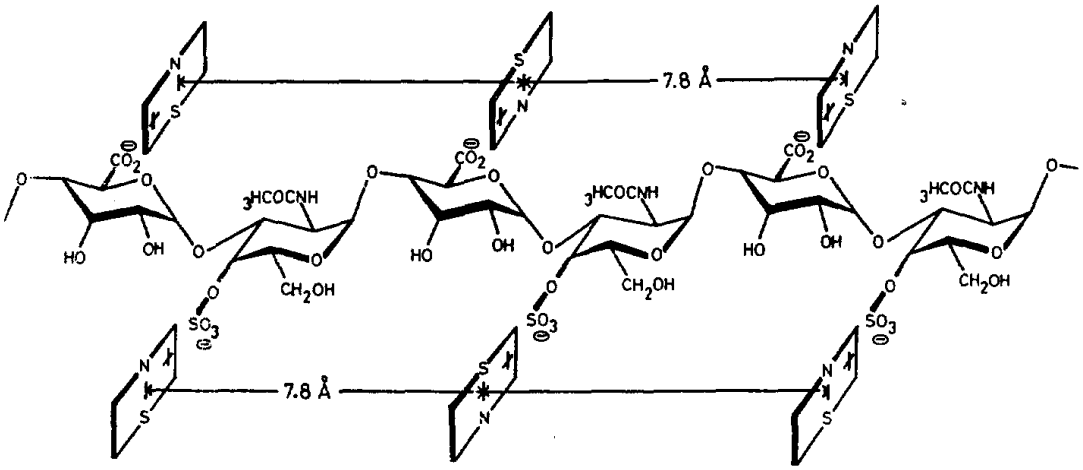


Figure No-3: Stacking model for the chondroitin sulfate - methylene blue complex

Assay of Marketed Formulation

Preparation of Test Solution

Twenty tablets were accurately weighed and crushed to obtain fine powder. An accurately weighed tablet powder equivalent to about 100mg of chondroitin sulfate was transferred to 100ml volumetric flask and sonicated for 15 minutes in about 50 ml of demineralised water and made up the volume with demineralised water. The resulting solution was filtered through Whatman filter paper. Volume of 5 ml of filtrate was further diluted to 100 ml with demineralised water.

Three 50 ml volumetric flasks were marked as standard, test and blank. Into each flask accurately 10 ml of methylene blue solution was transferred. 2ml of standard solution was pipette into standard flask, 2ml of test solution into test flask and 2ml of water into blank volumetric flasks. The volumes were made up to 50ml with demineralised water and shaken well for 30 minutes by inversion of the volumetric flasks at 5 minutes intervals. The absorbance of reagent blank, standard and test solutions were measured at 664 nm using water as blank. The absorbance value of standard and test was obtained after subtraction from the reagent blank value. The amount of chondroitin sulfate was calculated using following formula.

Calculation: The formula gave assay value of chondroitin sulfate in mg / tablet

$$\frac{A_{Blank} - A_{Test}}{A_{Blank} - A_{Std.}} \times \frac{Wt_{Std}}{100} \times \frac{5}{100} \times \frac{2}{50} \times \frac{100}{Wt_{Test}} \times \frac{100}{5} \times \frac{50}{2} \times \frac{P}{100} \times W_{Ave}$$

Where,

A_{Blank} = *Absorbance of reagent blank (Methylene blue)*
A_{Test} = *Absorbance of the chondroitin sulfate in the sample.*
A_{Std} = *Absorbance of the chondroitin sulfate in the standard*
$Wt._{Std}$ = *Weight of the chondroitin sulfate working Reference standard in mg*
$Wt._{Test}$ = *Weight of the sample taken in mg*
P = *Percent purity of chondroitin sulfate working reference standard (92.42 %)*
W_{Ave} = *Average weight of the tablet in mg*

Method Validation

Validation is the process of established documented evidence which provides high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality attributes. The method was validated by various parameters as per ICH guidelines (table no 2)

Linearity

Varying concentrations of chondroitin sulfate were treated with methylene blue within the range 0.5 µg/ml to 4 µg/ml corresponding to 50%, 75%, 100%, 125%, and 150% of chondroitin sulfate. The linearity of chondroitin sulfate was found to be 0.5 - 4 µg/ml and linear regression was found to be $r^2 = 0.9985$.

Accuracy

Accuracy of the method was determined in terms of % recovery of standard chondroitin sulfate at three different concentrations (50%, 100%, and 150%). Result of the recovery study were found to be within the acceptable criteria 100 ± 10 %, indicates sensitivity of the method towards detection of chondroitin sulfate and non interference of excipients in the method (table no1).

Table No –1: Recovery Calculation for chondroitin sulfate

Spike level	Theoretical Value (mg/ml)	Practical Value (mg/ml)	Recovery (%)	Average	RSD (%)
Level-I				98.95	0.207
50 % -1	0.502	0.4973	99.06		
50 % -2	0.511	0.5045	98.72		
50 % -3	0.497	0.4925	99.09		
Level-II				100.05	1.71
100 % -1	1.007	1.0188	101.17		
100 % -2	1.019	0.9995	98.08		
100 % -3	1.012	1.0212	100.9		
Level-III				100.20	0.823
150 % -1	1.511	1.5017	99.38		
150 % -2	1.503	1.5185	101.03		
150 % -3	1.508	1.5113	100.2		

System Precision

The precision of the system was determined by 6 repetitive absorbance of the same standard solutions by using 2 ml of stock solution. As the value of % RSD of system precision study were within the acceptable limit (less than 2%). Hence the method provides good precision.

Method Precision

The precision of the method for the assay of chondroitin sulfate was determined by the assay of six aliquots of the homogeneous sample. As the value of % RSD of system precision study were in within the acceptable limit (less than 2%). Hence the method provides good precision and reproducibility.

Specificity
The study was conducted to prove that the absorbance obtained in the samples is only due to chondroitin sulfate without any interference from other excipients. Placebo solution at varying concentration does not show any absorbance at 664 nm. Hence the method is specific for the determination of chondroitin sulfate.

Solution Stability
The stability of the analytical solution for assay of chondroitin sulfate was determined by the assay of sample preparation at fixed intervals of time. The % RSD for the assay values for chondroitin sulfate up to 24 hours was 1.140. This indicates that the analytical solution was stable up to 24 hours.

Ruggedness
The ruggedness of the method for assay of chondroitin sulfate was determined by the assay of six aliquots of the homogeneous sample on different instrument and by different analyst. The % RSD for the assay values for chondroitin sulfate in ruggedness study was found to be 0.0035. This indicates that the method has good reproducibility and very less random error.

The results of the method developed and validated for chondroitin sulfate in formulation are depicted in the table no 2. The results developed method showed good agreement with the labeled claim in the formulation analyzed.

Table No- 2: Summarised tabulated results for validation of the analytical method for chondroitin sulfate

Parameter	Acceptance criteria	Results Obtained
1. Linearity	Regression coefficient (r^2) not less than 0.999 Beer's Range Regression Equation	0.998 0.5 – 4 µg/ml y = 0.0287 x+0.0028
2. Accuracy	Recovery between 98 – 102 %	99.72 -100.17 %
3.System precision	% RSD should be less than 2.0 %	1.21 %
4. Method precision	% RSD should be less than 2.0 %	1.08 %
5. Specificity	Non interference of placebo and blank in analysis	Complies
6. Ruggedness	% RSD should be less than 2.0 %	0.0035 %
7.Sandell's sensitivity (mg/ml/0.001 abs units)	----------	0.00471

RESULTS AND DISCUSSION

The proposed spectrophotometric method has been applied to quantify chondroitin sulphate by interacting with methylene blue and based on the principle of decrease in the absorbance of methylene blue at 664 nm upon complexation with chondroitin sulfate. The Beer-Lambert's law obeyed in the range of 0.5 - 4 µg/ml with correlation coefficient at 0.9985. The accuracy of the method was determined at 50%, 100% and 150% level and the % recovery ranged from 99.72 - 101.17 %. The % RSD less than 2% indicates the method was accurate and precise. The method was found to be sensitive with respect to Sandell's sensitivity.

Hence developed method was found to be simple, accurate, precise, reliable and reproducible for routine quantitative estimation of chondroitin sulfate, which can be adopted in Quality control laboratories.

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