IJPSR (2014), Vol. 5, Issue 11 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 08 April, 2014; received in revised form, 23 May, 2014; accepted, 11 July, 2014; published 01 November, 2014

A pH-GRADIENT SAX-HPLC METHOD FOR DETERMINATION OF TRACE LEVELS OF OVER SULFATED CHONDROITIN SULFATE AND DERMATAN SULFATE IN HEPARIN SODIUM

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Keywords:

Lithium perchlorate in situ, pH-gradient, SAX HPLC, Heparin, Dermatansulphate (DS), Oversulphated chondroitin sulphate (OSCS), SAX HPLC

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ABSTRACT: A rapid and sensitive pH-gradient SAX HPLC method for the determination of Dermatan sulphate (DS) and trace levels of Oversulphated chondroitin sulphate (OSCS) in Heparin sodium was developed and validated. A pH gradient was opted to strike a balance between resolution and sensitivity because the response of OSCS was found to be better at neutral pH and the resolution between DS and Heparin was good at acidic pH. The use of commercially available lithium perchlorate was avoided and an in situ preparation of the same was opted to decrease the baseline drift which is a major concern for gradient methods using perchlorate mobile phases for compounds being analyzed at lower wavelengths. Chromatographic separation was achieved using a hydrophilic polymer resin-based SAX column, a linear gradientelution from 15% to 70% of solution-B which is 1.0M lithium perchlorate containing 5mM sodium dihydrogen phosphate at pH 6.0 and solution-A being 5mM sodium dihydrogen phosphate at pH 3.0 and a UV detection at 202 nm. The method has been validated and the limit of detection for DS and OSCS were found to be 15µg/ml and 20µg/ml respectively which is 0.03% &0.04% respectively with respect to the method concentration of 50 mg/mL. The method was found to be linear over the range of 50-150% of the specifications of the impurities with respect to the working sample concentration. The precision, accuracy, specificity robustness and solution stability were determined.

INTRODUCTION: Heparin sodium is the sodium salt of sulfated glycosaminoglycans containing a heterogeneous mixture of repeating uronic acid and N-sulphated glucosamine groups of varying molecular weights. The average molecular weight of heparin is about 15000 Daltons and its biological activity helps in delaying the clotting of blood. Heparin is usually isolated from the porcine or bovine intestinal mucosa and hence is prone to contamination and there is a need to monitor and control the potential impurities during analysis of Heparin[1-4].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.5(11).4916-24

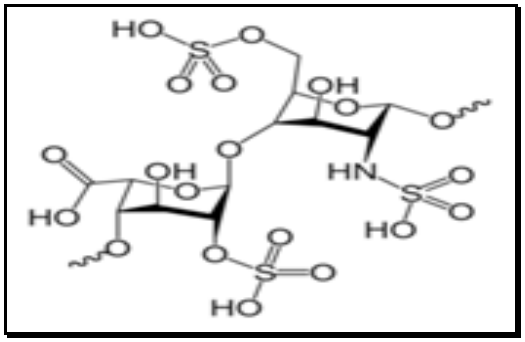
Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.5(11).4916-24

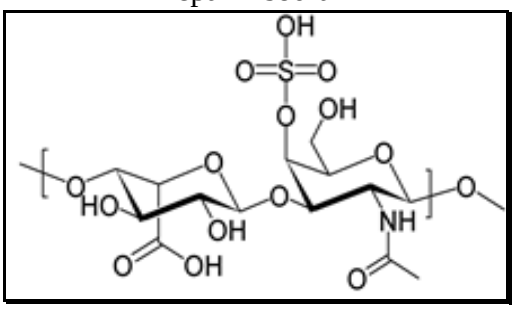
The major impurities (**Fig. 1**) which have to be monitored are Dermatan sulfate (DS) - a process related impurity of Heparin and over sulfated chondroitin sulfate (OSCS) - a contaminant or an adulterant which was intentionally mixed to reduce the cost of production and lead to several deaths in USA in 2008 [5, 6].

The recent guidance for industry published by the Food and Drug Administration (FDA), U.S. Department of Health and Human Services for monitoring the quality of crude heparin also insists the need for monitoring the potential contaminant OSCS in Heparin[7].

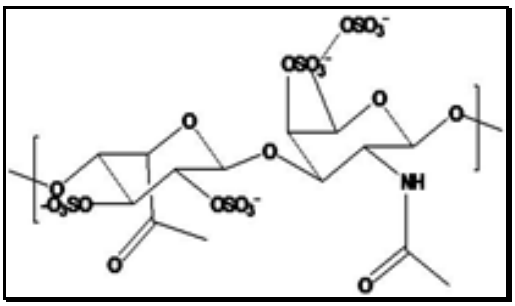
Fig. 1 Chemical Structures of Heparin sodium, Dermatan sulfate and over sulphated Chondroitin sulphate.



Heparin sodium



Dermatan sulfate



Oversulfated chondrotin sulfate

We have been continuously working on the development of analytical methods for Heparin[8-11] and there are several other methods in the literature using SAX HPLC for the oligosaccharide chain mapping of depolymerized heparins[12-14]. The tests present in the USP and EP monographs are either limit tests which do not quantify the amount of the impurities or adopt a degradation process prior to analysis [3, 4].

There are literature methods[15, 16] for Crude Heparin recommended by the recent FDA guidance - one is a colorimetric microplate assay method and the other is a SAX HPLC method which uses a high salt concentration of up to 2.5 M sodium chloride in the mobile phase, a polyvinylbenzyl ammonium (divinyl benzene) resin based SAX column with medium-low hydrophobicity and the LOD of OSCS by this method is less than 0.1%[7]. There is another method in the literature for determination of OSCS in Heparin API using CD (Circular dichroism) detection and the LOD of OSCS by this method is stated as 0.22 mg/ml[17].

Though the crude Heparin is being monitored and screened for the potential impurities as per the guidance for industry, it is equally important to monitor the Heparin API and the amount of OSCS present in the API would be very low. Hence to monitor trace levels of OSCS in Heparin API we conducted further method development experiments to produce a more sensitive method especially for determination of the potential contaminant, OSCS. We found that the peak response of OSCS was high at neutral pH, whereas the resolution between DS and Heparin was obtained only with an acidic pH (most of the existing methods opt for an acidic pH).

Therefore a pH gradient method was developed to achieve better resolution and sensitivity. Another important factor was the use of lithium perchlorate (which is prepared in situ by treating lithium hydroxide with perchloric acid) mobile phase which decreased the drift in the baseline significantly facilitating better limit of detection and also improved the resolution between Heparin and OSCS. The LOD of OSCS in this method was 0.04% (i.e. 20µg/ml) with respect to the method concentration of 50mg/ml.

The HPLC method developed employs a strong anion exchange column containing a hydrophilic polymer resin chemically bonded with quaternary ammonium functional groups and a gradient elution of 1.0 M lithium perchlorate (in situ) mobile phase containing 5mM phosphate buffer and adjusted to a pH of 6.0 (solution-B) and 5mM phosphate buffer at pH 3.0 (solution-A). A UV detector set at 202 nm was used and the sample solutions are prepared in water. The sample concentration is 50 mg/ml and an injection volume of 50µl is used. The analytical method was validated and was found to be precise, accurate and linear over a range of 50-150% of the individual specifications of the impurities.

MATERIALS AND METHODS:

Reagents and materials

Heparin sodium samples were obtained from Gland Pharma Limited, Dundigal, Hyderabad.

Dermatan sulphate and Over sulphated chondroitin sulphate USP reference standards, Sodium hydroxide pellets, extrapure, Lobachemie, Sodium dihydrogen phosphate monohydrate GR, Merck, Lithium hydroxide anhydrous, Laboratory grade, Fisher Scientific, Perchloric acid about 70%, ACS, Merck and Orthophosphoric acid, HPLC grade, Fisher Scientific were used.

Instrumentation

A Shimadzu LC 2010A model HPLC equipped with gradient pump, eluent degasser, autosampler, column oven and UV detector was used. Data acquisition was performed with a Shimadzu LC solutions software/chromatography data processor (Shimadzu Corporation, Japan).

Chromatographic conditions

A Glycomix^{TM} SAX column (250mm length x 4.6mm id) and guard column (50mm length x 4.6mm id) manufactured by Sepax Technologies Inc. were used for the determination of contents of Dermatan sulphate and Oversulphated chondroitin sulphate in Heparin sodium. The mobile phase was a mixture of solution A - 5mM sodiumdihydrogenphosphate monohydrate (pH adjusted to 3.0 with phosphoric acid) and solution B - 1M lithium perchlorate (prepared in situ) containing 5mM sodium dihydrogen phosphate monohydrate (pH adjusted to 6.0 with sodium hydroxide or phosphoric acid).

A linear gradient from 15 to 70% of solution B was used over a period of 22 minutes, return to 15% solution B at the end of 23 minutes and a stabilization period of 12 minutes at 15% solution B, thereby resulting in a 35 minutes runtime. The flow rate was 0.6 ml/min. The column temperature was maintained at 30°C. The sample concentration was 50 mg/ml in water, injection volume was 50 µL and the wavelength of detection was 202 nm.

Solution preparation

Mobile phase preparation:

Solution A:

Accurately weigh about 690 mg of sodium dihydrogenphosphate monohydrate, dissolve and make up to 1000 ml with MQ water and adjust the pH to 3.0 with dilute phosphoric acid solution. Filter the solution through a 0.45µ membrane and sonicate to degas.

Solution B: Accurately add about 86 ml of 70% perchloric acid to about 250 ml of Milli-Q water and cool the solution. Accurately weigh about 24 g of anhydrous lithium hydroxide, add about 500 ml of Milli-Q water and cool the solution. Add the cooled perchloric acid solution slowly and drop wise to the cooled lithium hydroxide solution and mix well. To this solution add about 690 mg of accurately weighed sodium dihydrogenphosphate monohydrate and sonicate to dissolve. Make up the volume to 1000 ml with Milli-Q water and adjust the pH to 6.0 with dilute phosphoric acid solution or sodium hydroxide (pellets or solution). Filter the solution through a 0.45µ membrane and sonicate to degas.

Diluent: Water

System suitability solution preparation

Prepare a 50mg/ml of Heparin sodium containing 0.5% each of DS and OSCS in water.

(Accurately weigh about 500 mg of Heparin sodium standard in a 10 ml volumetric flask, add 1.0 ml of 2.5mg/ml OSCS standard solution and 1.0 ml of 2.5mg/ml DS standard solution, dissolve and dilute to volume with water)

Diluted Standard preparation

Prepare a solution containing 0.25mg/ml each of DS and OSCS in water.

(Accurately pipette out 1.0 ml of 2.5mg/ml OSCS standard solution and 1.0 ml of 2.5mg/ml DS standard solution into a 10 ml volumetric flask and dilute to volume with water)

Sample preparation

Prepare a 50mg/ml solution of Heparin sodium API sample in water.

(Accurately weigh about 500 mg of Heparin sample in a 10 ml volumetric flask, dissolve and dilute to volume with water)

RESULTS AND DISCUSSION:

Development of chromatographic method

Optimization of mobile phase:

Choice of perchlorate:

The use of Perchlorate salt in the mobile phase is known to enhance the UV absorption of compounds which do not have significant wavelength of absorption. During our initial method development experiments we had used sodium perchlorate[9] in the mobile phase and later we had also used ammonium perchlorate[11]. Sodium perchlorate is also being used in the USP test method[3] to establish the "absence of Oversulphated chondroitin sulphate". When we were working further to develop a method to overcome the high drift in the baseline we found that the in situ preparation of perchlorate mobile phase[10, 11] brought down the high drift in the baseline from 75-100 mV to less than 25 mV. As a part of the method development protocol we evaluated different perchlorate salts and method development experiments were performed using sodium, ammonium and lithium perchlorate in the mobile phase. The other perchlorate salts such as potassium perchlorate and cesium perchlorate could not be evaluated during method development due to their poor solubility in water. After several trials lithium perchlorate was found to be the most suitable as it yielded a high resolution between Heparin and OSCS when compared to sodium perchlorate and ammonium perchlorate (**Fig.2**). The limit of detection for OSCS was enhanced significantly with lithium perchlorate mobile phase when compared to the other perchloratemobile phases (**Table 1**).

TABLE 1 COMPARISON OF LIMIT OF DETECTION OF DS & OSCS AND RESOLUTION WITH DIFFERENT PERCHLORATE MOBILE PHASES

Mobile phase (Solution B)	LOD (with respect to method concentration)		Resolution	
	DS	OSCS	between DS and Heparin	between Heparin and OSCS
1.0M $NaClO_4$	0.03%	0.08%	1.81	1.94
1.0M NH_4ClO_4	0.03%	0.06%	1.95	2.01
1.0M $LiClO_4$	0.03%	0.04%	2.25	3.33

The baseline drift was found to be very high when commercial perchlorate salts were used and the in situ preparation of perchlorate was found to considerably decrease the baseline drift (**Fig. 3**). However the resolution yielded by a 1.2M concentration of the commercial salt of lithium perchlorate was equivalent to that obtained with a 1.0M concentration of the in situ preparation.

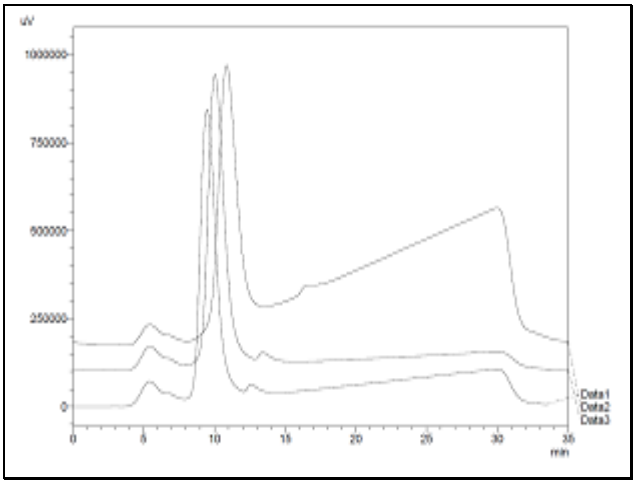


FIG. 2 COMPARISON OF SYSTEM SUITABILITY CHROMATOGRAMS OBTAINED WITH $NaClO_4$ (IN SITU), NH_4ClO_4 (IN SITU) AND $LiClO_4$ (COMMERCIAL) MOBILE PHASES

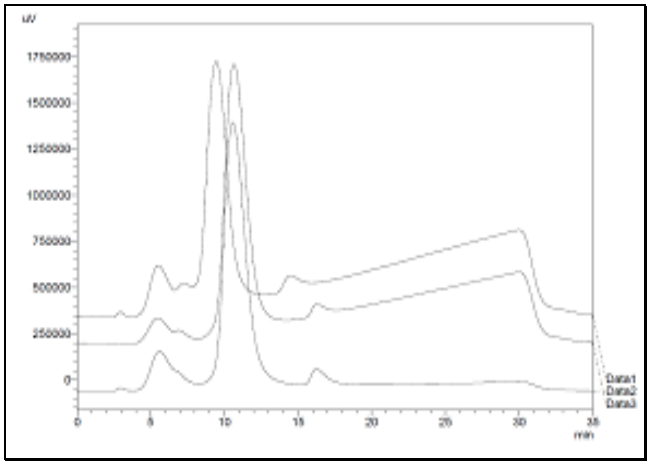


FIG. 3 COMPARISON OF BASELINE DRIFTS IN SYSTEM SUITABILITY CHROMATOGRAMS OBTAINED WITH $LiClO_4$ - COMMERCIAL AND $LiClO_4$ - IN SITU MOBILE PHASES

Choice of pH:

In our previous method[11] we used ammonium perchlorate as mobile phase and the pH of both solution A and B was 3.0. However the main objective was to improve the sensitivity of the method with respect to determination of OSCS. When different pH values (ranging from 3 to 6) were evaluated OSCS response was found to

increase with increase in pH, but the response of DS was not varying with change in pH (**Fig.4**). However the resolution between DS and Heparin was decreasing with increase in pH and the resolution between OSCS and Heparin was not affected by change in pH. Therefore a pH gradient from 3 to 6 was opted because a pH of 3 is required for separation of DS and heparin and a higher pH is required to achieve a better response for OSCS (**Fig.5**)

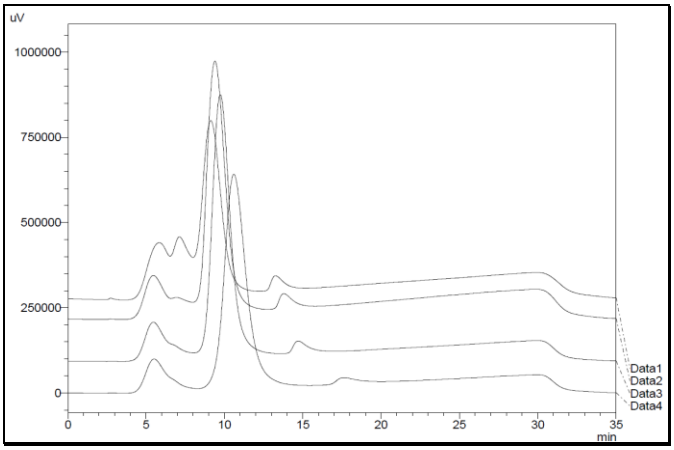


FIG. 4 COMPARISON OF SYSTEM SUITABILITY CHROMATOGRAMS– MOBILE PHASE pH 3.0, 4.0, 5.0 & 6.0

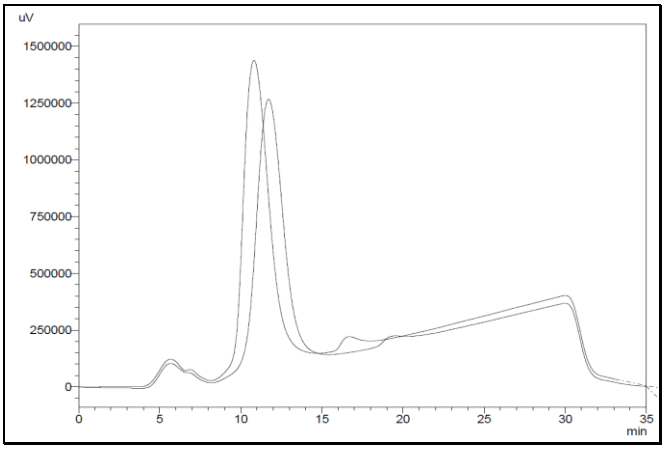


FIG. 5 COMPARISON OF SYSTEM SUITABILITY CHROMATOGRAMS– MOBILE PHASE pH 3.0 Vs. pH GRADIENT

Choice of Column:

On evaluation of strong anion exchange columns from different manufacturers, it was found that a hydrophilic polymeric substrate or resin (on which the quarternary ammonium functional groups are chemically bonded) yielded better resolution of analytes. Hydrophobic substrates yielded broad peaks resulting in longer runtimes and decreased sensitivity of impurities, whereas the column life was too short for silica based stationary phases. Hence Glycomix^{TM} SAX column from Sepax Technologies Inc. containing a hydrophilic polymer resin chemically bonded with quartenary ammonium functional groups was employed.

Gradient optimization:

Several trials were performed to optimize the gradient program as it was very critical to achieve a good resolution between DS and Heparin at a shorter run time. The initial percentage of 15% solution-B in the gradient program is very critical as an initial 10% or less resulted in broader peaks with no improvement in resolution and an initial 20% or greater resulted in decrease in resolution. The runtime of the previously published methods in the literature varies from 40 to 75 minutes. However with the optimized linear gradient of 15-70% of solution B the run time was 35 minutes.

Diluent optimization:

Originally the diluent was 15:85 solution B:A which is also the initial composition of solution A and B at the start of the gradient program, but the pH of both solution A and B was 3.0[11]. In the newly optimized method a pH gradient is being employed in which the pH of solution-A and solution-B are different, hence it is not possible to use the same diluent as the pH of diluent will be different from that of the mobile phase. Therefore water is chosen as the diluent, but the only disadvantage in using water as the diluent would be a split peak for DS due to its complex nature (refer typical chromatogram of DS in (**Fig.10**). However if both the peaks are integrated together the response of DS was found to increase linearly with concentration. Hence water was finalized as the diluent as it is always better than dissolving the compound in buffer and it was decided to integrate the two peaks of DS together in the diluted standard chromatogram.

Method validation

The optimized SAX-HPLC method was validated according to ICH guidelines[18] with respect to range, accuracy, precision, linearity, specificity, limit of detection, limit of quantification, accuracy at LOQ and robustness. System suitability parameters were also evaluated.

System suitability test

The acceptance criteria set was: the resolution between dermatan sulphate and heparin should be not less than 1.5 and the resolution between heparin and over sulphated chondroitin sulphate should be not less than 2.0. The results obtained were within acceptable limits. Typical system suitability chromatogram is as shown in **Fig.6**.

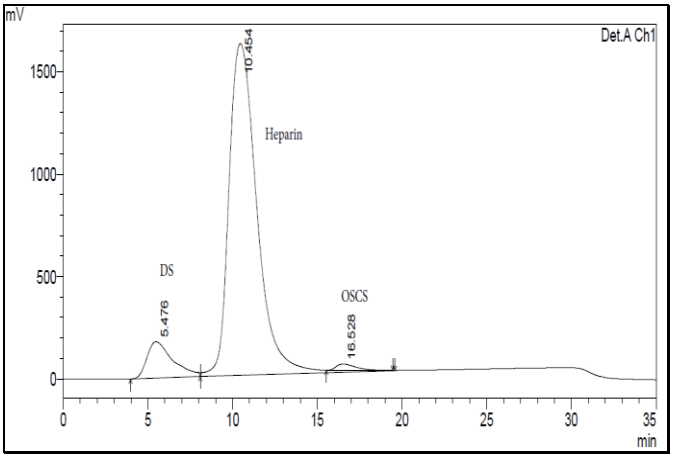


FIG. 6 TYPICAL SYSTEM SUITABILITY CHROMATOGRAM

Range (Accuracy, Precision & Linearity)

Accuracy:

Recovery solutions were prepared at concentrations spanning from 50 to 150% of the proposed specification of the impurities with respect to the test concentration of 50.0 mg/ml. The % recovery for DS and OSCS were calculated for the individual runs at each level and a mean of the recovery was determined for each impurity based on the un-rounded data and reported to one decimal place. The accuracy (recovery) results are presented in **Table 2**. The overlay of range chromatograms is presented in **Fig.7**. Accuracy at each level was within the acceptance criteria of 80.0 – 120.0 % of the theoretical concentration.

TABLE 2: RESULTS OF ACCURACY STUDY

Level	Accuracy (% recovery)	
	DS	OSCS
50%	111.4	93.6
75%	110.4	82.2
100%	110.7	87.3
125%	108.9	90.6
150%	108.0	93.6

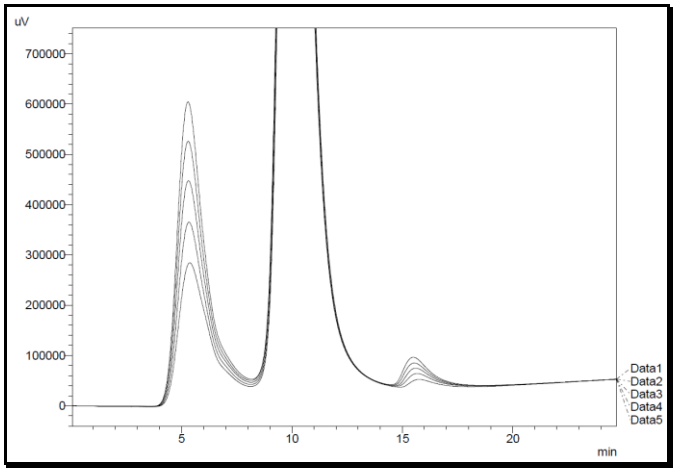


FIG. 7 OVERLAY OF CHROMATOGRAMS OF HEPARIN SPIKED WITH DS AND OSCS OVER THE RANGE ESTABLISHING PRECISION, ACCURACY AND LINEARITY

Precision:

Repeatability was determined by analyzing the sample preparation containing Heparin sodium at the method concentration and the impurities DS and OSCS spiked at 100% level of the specification with respect to the method concentration. The Heparin sodium API control sample without any spiking of impurities was analyzed to obtain the basal levels of the impurities. The content of related substances (DS and OSCS) was determined for each of the run and the mean of the related substances were determined. As shown in **Table 3** percent RSD for the related substances results of six runs (containing impurities DS and OSCS at the 100% spiked level) was 0.5% and 0.4% for DS and OSCS respectively. Therefore, the method is precise and repeatable.

TABLE 3: PRECISION RESULTS

No	Level	Run	DS (%)	OSCS (%)
1	100%	1	2.31	0.42
2	100%	2	2.31	0.42
3	100%	3	2.31	0.42
4	100%	4	2.30	0.42
5	100%	5	2.29	0.41
6	100%	6	2.29	0.41
Average			**2.30**	**0.41**
% RSD			**0.5**	**0.4**

Linearity:

Linearity was performed as a part of range study to assess whether a linear relationship is obtained between the response and the concentration for the impurities over the intended operating range of the method. A minimum of five concentration levels were analyzed over a range of 50 to 150% of the proposed specification of each of the impurities. A linear regression analysis (without forcing through the origin) was performed on the data (concentration and peak response). The individual data points (**Table 4 & 5)** and plots of response versus concentration (**Fig. 8 & 9**) are presented for impurities DS and OSCS. Index of determination (r^2) was found to be 0.9999 and 0.9906 for impurity DS and OSCS respectively.

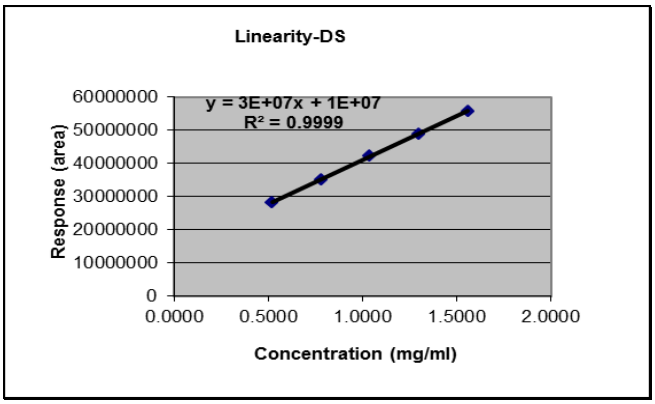


FIG. 8 LINEARITY PLOT OF DS

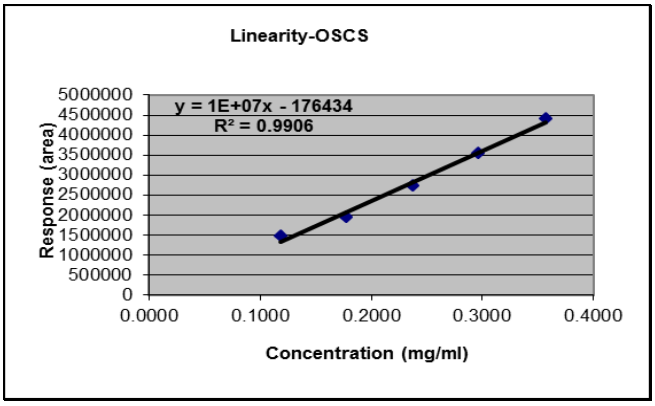


FIG. 9 LINEARITY PLOT OF OSCS

TABLE 4: LINEARITY RESULTS OF DS

Level (with respect to specification)	Concentration in % (with respect to method concentration)	Concentration in mg/ml	Response (Area)
50 %	1.0%	0.5200	28194742
75 %	1.5%	0.7800	35155178
*100%	2.0%	1.0400	42075980
125%	2.5%	1.3000	48821105
150%	3.0%	1.5600	55554490

TABLE 5: LINEARITY RESULTS OF OSCS

Level	Concentration in % (with respect to method concentration)	Concentration in mg/ml	Response (Area)
50 %	0.250%	0.1188	1464995
75 %	0.375%	0.1781	1931494
*100%	0.500%	0.2375	2734934
125%	0.625%	0.2964	3541298
150%	0.750%	0.3572	4407404

*100% specification level of DS and OSCS with respect to method concentration respectively

Specificity

Specificity was evaluated to ensure that no other compounds that may be present interfere appreciably with the quantitation of the analyte. Specificity of the method was demonstrated by its ability to separate Heparin sodium from its impurities. To demonstrate that the related substances/known synthetic impurities do not interfere with the quantitation of Heparin sodium, the drug substance and the impurities DS and OSCS were analyzed individually and their retention times were compared to establish separation. It was found that there were no peaks due to the diluent-blank or the impurities interfering with the quantification of Heparin sodium. (**Fig.10**)

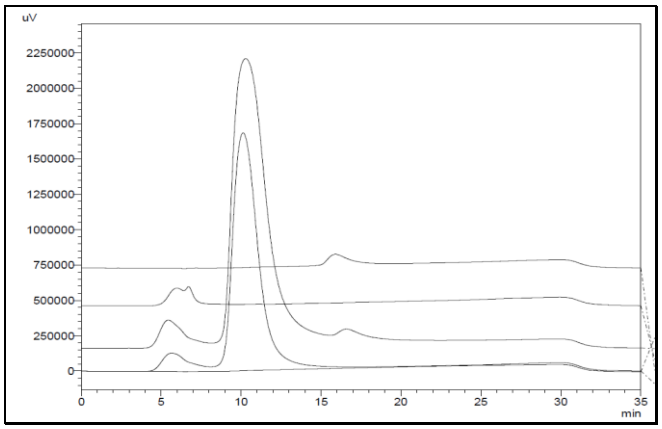


FIG.10 OVERLAY OF CHROMATOGRAMS FROM SPECIFICITY STUDY

Limit of detection and Limit of quantification:

The limit of detection and limit of quantification was established individually for the impurities (DS and OSCS) by analyzing the solutions prepared at

different concentrations, ranging from 1.0% to 0.005% with respect to the test concentration of 50.0 mg/ml. The overlay of LOD chromatograms for DS and OSCS are presented in **Fig.11&12**.

The limit of detection and limit of quantification of DS and OSCS were determined using the slope method. The LOD and LOQ obtained for DS were 0.032% and 0.099% respectively. The LOD and LOQ obtained for OSCS were 0.043% and 0.131% respectively. A solution containing DS and OSCS was prepared around its LOQ level concentration and injected in six replicates. The RSD value obtained for the area of DS and OSCS at LOQ were 0.8% and 1.4% respectively. Recovery solutions of Heparin sodium spiked with the impurities DS and OSCS at their respective LOQ levels were prepared and analyzed to establish the accuracy at LOQ. The percent recovery for DS and OSCS at their corresponding LOQ levels were 112.8% and 111.9% respectively which was within the acceptance criteria of 70.0 – 130.0% of the theoretical concentration at the LOQ level.

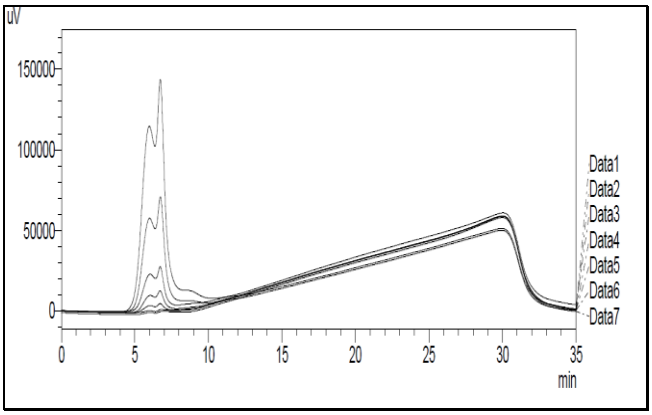


FIG.11 OVERLAY OF LOD CHROMATOGRAMS OF DS

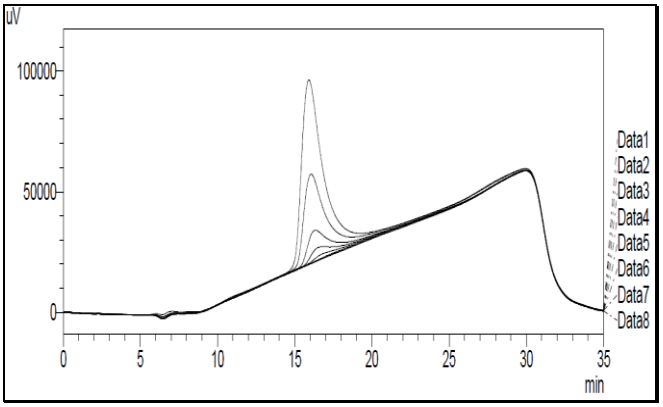


FIG.12 OVERLAY OF LOD CHROMATOGRAMS OF OSCS

Robustness:

The method robustness measures the ability of the analytical method to tolerate minor variations in the method recommended parameters, demonstrating the reliability of the method under normal use. In order to demonstrate robustness a few of the parameters such as column temperature, mobile phase pH, and flow rate was varied intentionally and the system suitability parameters were evaluated with each variation. The system suitability criteria was met with all the variations; the method was found to be robust with variations of ± 5^{o}C in the column temperature, robust with variations of ± 0.5 in the pH of the mobile phase (both solution-A and solution-B were evaluated individually) and robust with ± 0.1 ml/minute flow variations in the mobile phase.

Solution stability:

One of the standard and sample preparations at 100% level of the method concentration was stored at room temperature and was analyzed periodically to establish the solution stability. The response of DS and OSCS peaks in the standard preparation and the content of the related substances in the sample solution were measured to establish the solution stability. The evaluations of the results obtained revealed that the standard solution and sample solutions are stable for a minimum period of 3 days when stored at room temperature.

CONCLUSION: The optimized pH gradient SAX-HPLC method using in situ lithium perchlorate mobile phase is a simple and high-sensitive analytical method for the quantification of trace levels of dermatan sulphate and over sulphated chondroitin sulphate in Heparin sodium. This method employs an appropriate pH gradient mobile phase and a suitable stationary phase to increase the sensitivity of the method and to improve the resolution between the analytes. The in situ preparation of lithium perchlorate reduces the high drift in the baseline and makes the method more economical as the commercial lithium perchlorate is expensive. The results of the validation study shows that this method can be used for the determination of contaminants and other impurities in Heparin sodium. This method may also be suitable for the crude Heparin if it is subjected to suitable treatment prior to analysis.

ACKNOWLEDGEMENTS: The author of this article sincerely thanks the management of Gland Pharma Limited for providing complete support to carry out this development and validation and also thanks JNTUH for giving an opportunity to pursue this research work.

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How to cite this article:
Kavitha G, Nadamuni G, Venkatesan CS**,** Mukkanti K and Raju VNSMVG: A pH-Gradient Sax-HPLC Method for Determination Oftrace Levels of Oversulfated Chondroitin Sulfate and Dermatan Sulfate in Heparin Sodium. Int J Pharm Sci Res 2014; 5(11): 4916-24.doi: 10.13040/IJPSR.0975-8232.5 (11).4916-24