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Method Development and Validation of Ion Chromatography Method for Determination of Free Sulfate in Fondaparinux Sodium Pre-Filled Syringe

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A simple ion chromatography method was developed for the quantitative determination of free sulfate in fondaparinux sodium pre-filled syringe for injection. Chromatographic separation was achieved on an anion-exchange resin column made of super macro porous polyvinyl benzyl ammonium polymer cross-linked with divinyl benzene (250 × 4.0 mm) with a mobile phase consisting of 60 mM carbonate buffer solution. Conductivity detector was employed with a flow rate of 0.7 mL min^{-1}, injection volume of 100 µL and column temperature of 30 ºC. Retention time of sulfate (SO_4^{2-}) was eluted at about 10.4 min. The developed method was validated in according to ICH Q2(R1) guideline and was found to be specific, precise, accurate, linear and robust. The precision was evaluated with six individual spiked samples of sulfate on Fondaparinux sodium for injection. The proposed method is linear ($r^2 > 0.9991$) and accurate, mean recoveries were 99.2-117.8 % at 3 different levels (50-150%). The robustness was performed by changing the flow rate of mobile phase (0.7 ± 0.1mL min^{-1}) and column temperature (30 ± 2 ºC). The proposed method is capable to determine free sulphate in fondaparinux sodium for injection in presence of excipients used in pharmaceutical formulation and also in its active pharmaceutical ingredient.

Keywords: Ion Chromatography, Sulfate determination, Fondaparinux sodium.

INTRODUCTION

Fondaparinux is a synthetic sulfated pentasaccharide, which binds antithrombin and inhibits the activated factor X (Xa). Fondaparinux is used as an anticoagulant medication and is chemically methyl O-2-deoxy-6-O-sulfo-2-(sulfoamino)-α-D-glucopyranosyl-(1→4)-O-β-D-glucopyranuronosyl-(1→4)-O-2-deoxy-3,6-di-O-sulfo-2-(sulfoamino)-α-D-glucopyranosyl-(1→4)-O-2-O-sulfo-α-L-idopyranuronosyl-(1→4)-2-deoxy-6-O-sulfo-2-(sulfoamino)-α-D-glucopyranoside, decasodium salt" with a molecular weight of 1728 g/mol and an empirical formula of $C_{31}H_{43}N_3Na_{10}O_{49}S_8$ (Fig. 1). Fondaparinux sodium injection, which is available in market as Arixtra® (by Glaxo-SmithKline), is a sterile and preservative-free injectable solution for subcutaneous use [1].

In this study, the authors aim to determine the amount of free sulfate impurity present in fondaparinux sodium injection (pre-filled syringes) using an ion chromatography as fondaparinux sodium contains eight sulfate units in its chemical structure [1]. As sulfate causes the cathartic effect in patients if it crosses the daily exposure limit [2]. According to the regulatory requirements, it is necessity to check the quality of drug and to ensure the safety of patients who have undergone for medication hence the amount of impurities (organic or inorganic) present in drug must be estimated quantitatively using the suitable technique. An ion chromatography is used to determine the free sulfate as it is one of a versatile techniques and mostly used to quantitatively determine the ionic compounds using the isocratic and/or binary gradient elution modes with suitable aqueous and non-aqueous mobile phases [3,4].

Literature survey reveals that no ion chromatography method was reported for the free sulphate determination either in drug substance or in formulated drug product of fondaparinux sodium except the USP monograph [5,6]. As specified in USP monograph of fondaparinux sodium, (a) Thermo Dionex Ion Chromatography system model ICS6000 and (b) Dionex ASRS 300-4 mm suppressor are compulsory required to perform the free sulfate determination, but these are very expensive and not readily available in an in-house laboratory (Table-1 for comparison between USP and proposed methods). Thus, alternatively, Metrohm

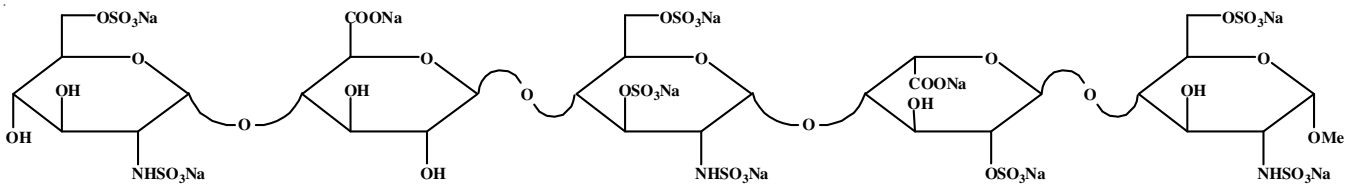

Fig. 1. Chemical structure of fondaparinux sodium

TABLE-1
COMPARISON b/w USP AND PROPOSED IC METHODS

Item	USP method	Proposed method
IC (Ion chromatography)	USP method works only when "Thermo Dionex IC system, model ICS6000" with at least binary pump is available.	The proposed IC method can work either with single pump or binary pump IC systems (*e.g.* Metrohm 930 Compact, IC Flex with auto sample Metrohm 863 Compact).
Suppressor	Dionex ASRS 300 4 mm suppressor is compulsory required to determine the sulfate.	No suppressor is required to determine the sulfate.
Mobile phase	3 mM carbonate solution (1 mM sodium carbonate and 2 mM sodium hydrogen carbonate) is recommded.	60 mM carbonate solution (10 mM sodium carbonate and 50 mM sodium bicarbonate) was used.
Elution	It can work only with Dionex columns.	It can work either with Dionex column or similar phase columns (*e.g.* Shodex IEC QA-825).
Capability	It can separate only sulfate peak in absence of formulation excipients (*e.g.* 0.9% sodium chloride).	It can separate the sulfate from blank peaks, placebo peaks (excipients without Fondaparinux sodium) without any interference.
Complexities	Negative peak is observed. The interference from placebo (chloride) peak was observed when the drug product was analyzed.	Negative peak and chloride peak were separated well even the formulated drug product was analyzed.

930 Compact IC Flex system (single pump) was selected and new, simple, rapid and reproducible ion chromatography method was developed for the quantitative determination of free sulfate in fondaparinux sodium (pre-filled-syringe) injection. The developed method was validated in according to the U.S Pharmacopeia [7] and ICH [8] for specificity, linearity, accuracy, precision, robustness and solution stability.

EXPERIMENTAL

Analytical grade sodium carbonate, sodium bicarbonate, sodium hydroxide and potassium hydroxide were purchased from Rankem, India. Sodium sulfate and sodium chloride were purchased from Standard Reagents, India. The analytical grade sodium hydroxide and HPLC grade acetonitrile were purchased from Finar, India. Samples of fondaparinux sodium API and fondaparinux sodium pre-filled syringe for injection and their placebo solutions were kindly supplied by Jodas Expoim Private Limited, Hyderabad, India.

The experiments of free sulfate determination were carried out on Metrohm 930 Compact IC Flex coupled with 863 compact autosampler (Metrohm Herisau, Switzerland). The out-put signal was monitored and processed using Metrohm′s software of MagIC Net 3.2 work station. Ultra-Sonic bath used for preparation of sample and standard solutions was from Labman, India. Standard weighing was done on the analytical balance (model: GH-252, make: AND, Japan). Ultrapure water (electrical resistivity ≥ 18.2 MΩ cm at 25 ºC) was collected from Millipore, Milli Q® IQ-7000 (Merck, France) water purification system.

Chromatographic system: A Dionex Ion Pac AS19 (length: 250 mm, diameter: 4.0 mm; an anion-exchange resin column made of super macroporous polyvinyl benzylammonium polymer cross-linked with divinyl benzene) was used for free sulfate determination in fondaparinux sodium pre-filled syringe for injection. The mobile phase used was a mixture of 10 mM Na_2CO_3 and 50 mM $NaHCO_3$ solution (60 mM carbonate buffer). Chromatographic detection was carried-out with conductivity detector in an isocratic elution mode and with flow rate of 0.7 mL min^{-1}, injection volume of 100 µL and column temperature of 35 ºC. The retention time of free sulfate (SO_4^{2-}) was 10.4 min and total run time of IC chromatogram selected was 20 min.

Standard solution: A standard stock solution (conc. 500 µg mL^{-1}) of sulfate was prepared in ultra-pure water using the commercially available sodium sulphate anhydrous 147.9 mg in 200 mL ultra-pure water. Using this stock solution, the final working standard solution was prepared to obtain the concentration 20 µg mL^{-1} and was subjected to the IC analysis of free sulfate determination in pharmaceutical formulation samples of Fondaparinux sodium pre-filled syringe for injection.

Samples: Each single dose of fondaparinux sodium, pre-filled syringe (PFS), affixed with an automatic needle protection system, contains 2.5 mg of fondaparinux sodium in 0.5 mL, 5.0 mg of fondaparinux sodium in 0.4 mL, 7.5 mg of fondaparinux sodium in 0.6 mL or 10.0 mg of fondaparinux sodium in 0.8 mL of an isotonic solution of sodium chloride and water for injection.

Sample solution: Ten pre-filled syringes of fondaparinux sodium for injection were opened and the sample solution was transferred in a suitable container and mixed well. A 4 mL of resulting pooled sample solution was transferred into a 5 mL volumetric flask and made up to the mark with ultrapure water. The concentration of Fondaparinux sodium is 4 mg/mL.

Method development trials: During the preliminary experiments, the ion chromatographic method [mobile phase: 3 mM

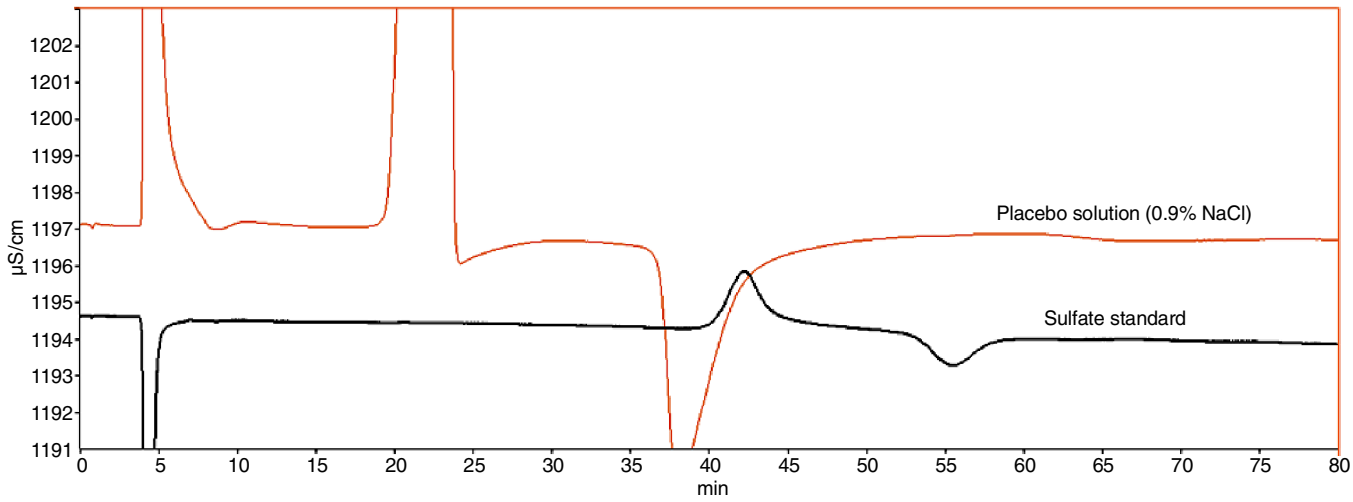

Fig. 2. A typical chromatogram of USP method generated in Metrohm IC

carbonate buffer (1 mM Na_2CO_3 + 2 mM $NaHCO_3$; conductivity: 200 µS; flow rate: 1.0 mL/min; injection volume: 50 µL; run time: 30 min; Dionex Ion Pac AS19, 250 × 4.0 mm] provided in the U.S Pharmacopeia was tried by analyzing the sulphate standard, placebo (0.9% NaCl) and sample of fondaparinux sodium for injection were injected and it was noticed that a negative peak of placebo solution was interfered with the retention time of the sulphate peak in standard solution (Fig. 2).

Due to the fact that many different buffers with different concentrations such as KOH (20-40 mM), NaOH (20-40 mM); Na_2CO_3 and $NaHCO_3$ (1-50 mM) (individually and combinely); 1 mM Na_2CO_3 with 5 % acetonitrile along with suppressor (100 mM sulphuric acid) and with-out suppressor were also tried to get the separation between negative peak of placebo and standard sulphate peak. In fact, afore-said trails were also conducted using the same stationary phase of Shodex IEC QA-825 (75 × 8.0 mm, 12 µm), but the desired separation was achieved with the following conditions (mobile phase: 10 mM Na_2CO_3 + 50 mM $NaHCO_3$; conductivity: 200 µS; flow rate: 0.70 mL/min; injection volume: 50 µL; run time: 30 min; column temperature: 35 ºC). The retention time of sulphate was found at 20 min (Fig. 3). Also, the same conditions were applied using Dionex Ion Pac AS19 (250 × 4.0 mm) and it

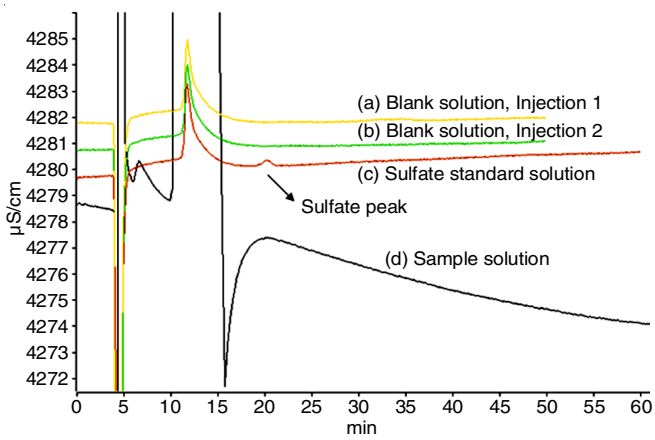

Fig. 3. Reference chromatogram of proposed IC method with Shodex IEC QA-825 (75 × 8.0 mm, 12 µm) column

was observed that the retention time of sulfate was found at 10 min without any interference of negative peak (Fig. 4). Thus, Dionex Ion Pac AS19 (250 × 4.0 mm) column was chosen as a final method condition because it provided a good peak response, early elution and shorter run time as compared to the Shodex IEC QA-825 column.

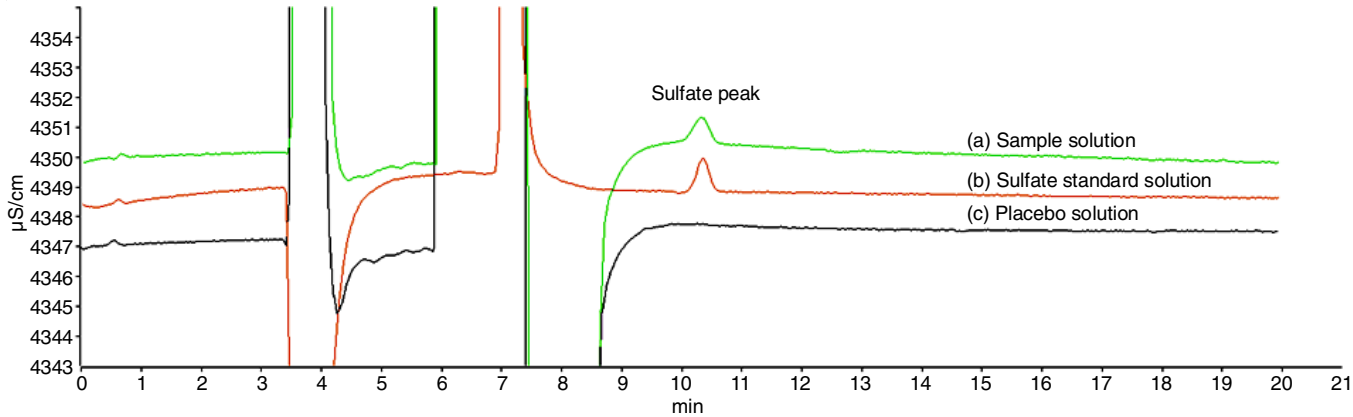

Fig. 4. A typical chromatogram of proposed IC method with Dionex, Ion Pac AS19 (250 × 4.0 mm) column

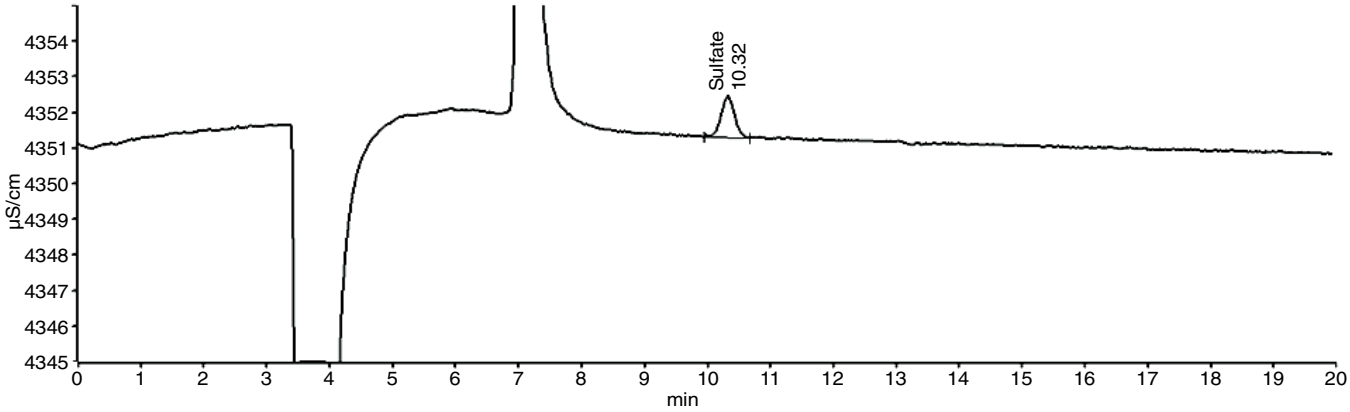

Fig. 5. A typical chromatogram of final optimized method

RESULTS AND DISCUSSION

The developed ion chromatography method of free sulphate was validated in according to USP and ICH Q2(R1) guidelines for system suitability, specificity, linearity, accuracy, precision, solution stability and robustness [7,8]. The main purpose of analytical method validation is to confirm the selectivity and suitability of the proposed ion chromatographic method to ensure safety, quality and efficacy of the drug.

System suitability: System suitability of the proposed method for free sulfate (20 µg mL^{-1}) was measured from the six replicate aspirations of standard (n = 6). System suitability parameters were expressed based on the percentage of relative standard deviation which obtained from the replicate aspirations of sulfate response (Table-2 and Fig. 5). A value of < 1.28% for %RSD (limit: ≤ 5%) indicates the precision and reproducibility of the sulfate response by the developed ion chromatographic method.

TABLE-2
SYSTEM SUITABILITY AND
PRECISION STUDY FOR SULFATE

S. No.	Sulfate peak area*	Recovery (%)**
1	0.2800	106.6
2	0.2841	104.8
3	0.2802	106.3
4	0.2836	103.0
5	0.2746	105.6
6	0.2835	105.9
Average	0.2810	105.4
% RSD	1.28	1.5

*Standard peak area obtained from the six replicate injections of standard solution; **Amount of sulfate recovery obtained from the sulfate spiked six individual samples of fondaparinux sodium for injection (pre-filled Syringe).

Specificity: Specificity of the developed test method was proven by determining the percent interference of diluent (ultrapure water) and placebo. No interference was found at retention time of the sulfate peak, this indicates that the excipients (0.9% NaCl) used in the pharmaceutical formulation did not interfere with the sulfate analyte. The reference chromatograms of specificity (selectivity) are shown in Fig. 4.

Precision: The precision of the developed ion chromatographic method was conducted by preparing and analyzing the six sulfate spiked sample solutions of fondaparinux sodium for injection at the target sample concentration (about 20 µg mL^{-1}). Percent content of sulfate in each spiked sample was calculated against the sulfate standard solution and the results are given in Table-2. The mean content of sulfate was found within the pre-defined limits. The percent relative standard deviation was 1.2.

Linearity: Linearity of the test method was evaluated by analyzed the standard solutions of sulfate with 5 different concentrations ranged from 5.014-40.109 µg mL^{-1}, which covered 25-200% of target concentration (20 µg mL^{-1}). The regression analysis was determined using linear regression: y = ax + b, where 'y' is the response of sulfate obtained from the standard solutions, 'a' is the slope of the regression line, 'x' is the concentration of sulfate, 'b' is the y-intercept of regression line. Fig. 6 showed that the regression parameters such as the correlation coefficient, slope and y-intercept of the calibration curve and residual sum of squares and the results are tabulated in Table-3. All the samples showed a linear behaviour in the chosen concentration ranges.

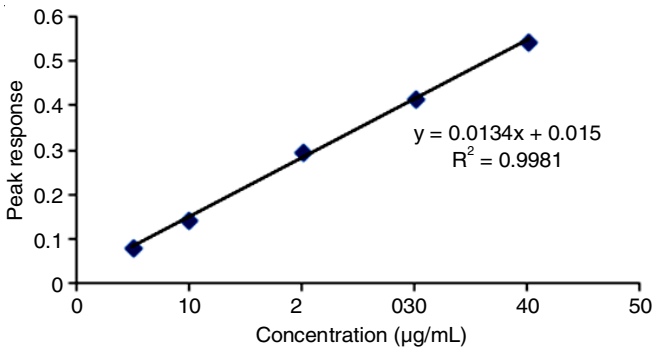

Fig. 6. Linearity graph of sulphate

Accuracy: Accuracy of the proposed method was evaluated by injecting the sulphate spiked sample solutions of fondaparinux sodium for injection at the concentration levels of 25, 100 and 150%. Percent recoveries were calculated by comparing with the standard responses and the results are summarized in Table-4. The mean recoveries of three spiking levels were

TABLE-4
RESULTS OF ACCURACY STUDY

Spike level	Prep.	Amount added (µg/mL)	Amount found (µg/mL)	Recovery (%)	Mean (%)	RSD (%)
LOQ (25%)	1	5.0137	5.7489	114.7	108.9	7.7
	2	5.0137	5.6485	112.7		
	3	5.0137	4.9747	99.2		
100%	1	20.0520	23.6120	117.8	108.8	7.6
	2	20.0520	20.3863	101.7		
	3	20.0520	21.4185	106.8		
150%	1	30.0813	33.3320	110.8	108.2	5.4
	2	30.0813	33.7979	112.4		
	3	30.0813	30.5436	101.5		

TABLE-3
RESULTS OF LINEARITY

Spike level	Concentration (ppm)	Area response
25%	5.0140	0.0796
50%	10.027	0.1425
100%	20.055	0.2972
150%	30.082	0.4163
200%	40.109	0.5461
Correlation coefficient (r)		0.9991
Intercept		0.0150
Slope		0.0134
y-Intercept at 100%		3.6

ranged from 99.2% to 114.7%. Each relative standard deviation was below 10%.

Solution stability: The prepared standard and sample solutions of sulfate were stored at room temperature (25 ± 2 ºC) in the analytical laboratory and found to be stable for 52 h. Thus, all the studies are preferred to run within 52 h of storing at about 23-27 ºC in the analytical laboratory.

Robustness: To validate the robustness of the developed method, deliberate variations are made in testing conditions of ion chromatographic method like changing the flow rate (0.7 ± 0.1 mL min^{-1}) and column temperature 30 ± 2 ºC. In all modified conditions, the retention time of sulfate peak was found well within the expected range (9 to 11 min) and thus, the developed method was found highly robust.

Conclusion

A new, simple, robust and isocratic ion chromatography method was developed and validated to determine the sulfate in fondaparinux sodium pre-filled syringe for injection. The proposed ion chromatographic method showed the specificity, accuracy (over a range 25-150% of target limit), precision (n = 6), linearity (over a range 25-200% of target limit), solution stability and robustness (change in flow rate 0.7 ± 0.1 mL min^{-1} and column temperature 30 ± 2 ºC). The challenges related to the interference faced during the method development and optimization was resolved appropriately. The developed method was also suitable for the estimation of free sulfate in APIs (active pharmaceutical ingredients) and can be used in routine and stability analysis for quality control in pharmaceutical industries.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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