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# Determination of endogenous glycosaminoglycans derived disaccharides in human plasma by HPLC: Validation and application in a clinical study

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**Abstract**

SB-424323 is a new, orally active anti-thrombotic agent presently in phase-II clinical development, with limited hemorrhagic risk and a unique mechanism of action involving the induction of glycosaminoglycans (GAGs) biosynthesis. The objective of the present study was to develop a simple and rapid high performance liquid chromatography (HPLC) method for determination of endogenous GAGs derived disaccharides in plasma samples from a phase-II clinical study of SB-424323. Sample preparation was a simple heat treatment of the diluted plasma followed by digestion of endogenous GAGs with chondroitinase ABC to yield unsaturated disaccharides, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S), and 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S). These disaccharides were recovered and purified using centrifugal filtration through a filter with 3000 molecular weight cut-off along with externally added internal standard 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-UA2S). A gradient reverse phase HPLC separation was developed on a Waters Symmetry $C_{18}$ column (4.6 mm × 150 mm, 5 μm) with a gradient mobile phase system consisting of 0.8 mM tetrabutylammonium hydrogen sulfate and 2 mM sodium chloride and acetonitrile at a flow rate of 1.0 mL/min. The eluate was monitored with an ultraviolet detector set at 230 nm. Plasma standard curves were linear ($r^2 \geq 0.994$) in the concentration range 1.0-20 μg/mL with a lower limit of quantification (LLOQ) of 1.0 μg/mL for each of the disaccharide. The mean measured quality control (QC) concentrations for the disaccharides deviated from the nominal concentrations in the range of −8.92 to 5.61% and −16.3 to 16.7%, for inter and intra-day, respectively. The inter and intra-day precision in the measurement of QC samples, were in the range of 3.21 to 18.2% relative standard deviation (R.S.D.) and 0.32 to 20.9% R.S.D., respectively. The inter and intra-day precision in the measurement of endogenous GAGs derived disaccharides in human control plasma, were in the range of 5.8 to 15.9% R.S.D. and 1.17 to 7.74% R.S.D., respectively. Stability of the processed samples was confirmed up to 48 h in the auto-sampler. The method is simple, reliable, and easily adaptable to analysis of large number of samples under logistics of a clinical study. The present method has been used to investigate the GAGs levels in the plasma of patients in a phase II clinical study of SB-424323.


*Keywords:* Glycosaminoglycans; Disaccharides; Biomarkers; Anti-thrombin; Pharmacodynamics

## 1. Introduction

SB-424323 is a novel, orally active anti-thrombotic agent presently in phase-II clinical development for prevention of thromboembolic events associated with atrial fibrillation and deep venous thrombosis. SB-424323 showed impressive anti-thrombotic activity in various preclinical models of venous thrombosis and moderate anti-thrombotic activity in arterial thrombosis models with minimal anticoagulant activity and bleeding risk. The unique anti-thrombotic mechanism of action of SB-424323, a β-D-xyloside is postulated to be via an elevation in circulating glycoaminoglycans (GAGs) and subsequent elevation of anti-thrombin activity via heparin cofactor II.

In vitro studies conducted using human cell lines showed that SB-424323 increased GAGs in culture medium in a concentration dependent manner, these results were consistent with

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other β-D-xylosides where they have been reported to substitute for xylosylated core proteins as initiators of synthesis of GAG free chains [1–4]. In vivo studies in rabbits with SB-424323 administered per orally, at a dose of 400 mg/kg resulted in a five-fold increase in plasma GAGs levels, these finding were similar in nature to another reported anti-thrombotic β-D-xyloside, Naroparcil [5–7].

GAGs are heterogeneous acidic polysaccharides, which circulate in plasma associated with a variety of other compounds, usually proteins and peptides [8–10]. GAGs have been associated with a number of physiological processes [11,12], however the exact physiological role of plasma GAGs is yet to be elucidated. The role of GAGs in maintaining the anti-coagulant and anti-thrombotic potential of human plasma is being increasingly studied and evaluated [13–20].

The low concentration of GAGs in plasma and poorly characterized interactions of GAGs with other plasma components makes it quite difficult to extract and purify these macromolecules. Accordingly, the reported methods for determination of endogenous GAGs in plasma require large volumes of plasma and are tedious with lengthy and complex purification steps [8,17,19,21–24]. Furthermore, these methods are single time point measurement of plasma GAGs, whereas our objective in the present study was to obtain an estimate of the plasma total GAGs over different time points during the course of treatment with SB-424323.

We required an assay method for determination of endogenous plasma GAGs, with low sample volume that could easily be adapted to a large number of samples with a rapid and simple sample preparation and low maintenance instrumentation. Recently, a number of methods were focused on the determination of exogenously administered GAGs with relatively smaller sample volume requirements. These considered indirect methods are based on the measurement of unsaturated disaccharides released quantitatively after depolymerization of GAGs (alternating copolymers of uronates and amino sugars) using specific enzymes. Indirect methods for measuring GAGs have become more prevalent, primarily because of their ability to handle disaccharides as small molecules in HPLC with resultant higher sensitivity [25–31].

In a recently reported indirect method for measurement of exogenously administered GAGs, microfiltration using commercially available centrifugal filters having a molecular cut-off of 5000 were used to recovery GAGs from plasma after protein digestion [28]. In this method, microfiltration was shown to be superior over conventional precipitation methods which are based on the use of cetylpyridinium chloride or ethanol/methanol [32]. In the present method, we have adapted the use of a filtration device after a simple plasma heat treatment to expose protein bound GAGs for enzymatic digestion with chondroitinase ABC. Subsequently, filtration was used to recover and purify released disaccharides. The filtered disaccharides in the sample were subjected to HPLC and the eluate monitored with an ultraviolet detector set at 230 nm.

In this method, we have waived the requirement of removal of GAGs from bound proteins by digestion with protease, which reduced the number of steps and increased the throughput. Furthermore, the method allowed us to use more relevant plasma disaccharide standards to evaluate the test sample concentration instead of solution standards. We report a simple and rapid method, which can be easily adapted to analyze a large number of samples under logistics of a clinical study. The present method has been used to investigate GAGs levels in plasma of patients in a phase II clinical study of SB-424323.

## 2. Experimental

### 2.1. Materials

Chondroitin sulfate A (CS-A), enzyme chondroitinase ABC, standard unsaturated disaccharides (Fig. 1), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-0S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S) and internal standard 2-acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-D-galactose (ΔDi-UA2S) were purchased from Sigma Chemical Co. (St. Louis, MO). Human control plasma (HCP) was purchased from Valley Biomedical Inc. (Winchester, VA). Tetrabutylammonium hydrogen sulfate was obtained from JT Baker (Phillipsburg, NJ). HPLC grade acetonitrile was purchased from Fisher Scientific (Newark, DE). Purified water from Barnstead Nanopure Diamond water systems (Dubuque, Iowa) was used for aqueous solutions and the mobile phase. Centricon YM-3 filter with molecular cut-off of 3000 were purchased from Millipore Corp. (Bedford, MA). All other chemicals used were of analytical grade.

### 2.2. Equipment and chromatographic conditions

The chromatographic system used was an Alliance, Waters 2695 Separation Module (Milford, MA). Chromatographic separation was achieved on a Symmetry C-18 analytical column (150 mm × 4.6 mm internal diameter, 5 μm particle size) from

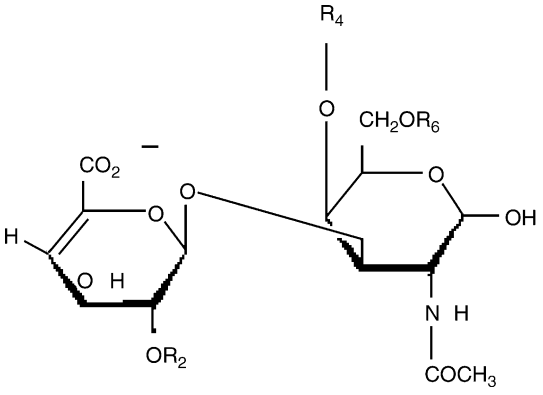


Fig. 1. Chemical structures of measured disaccharides (all are sodium salts): ΔDi-0S, ΔHexAGalNAc, $R_2$ = H, $R_4$ = H, $R_6$ = H; ΔDi-6S, ΔHexA-GalNAc (6-$OSO_3$), $R_2$ = H, $R_4$ = H, $R_6 = SO_3^-$; ΔDi-4S, (HexA-GalNAc (4-$OSO_3$), $R_2$ = H, $R_4 = SO_3^-$, $R_6$ = H; ΔDi-UA2S, ΔHexA-GalNAc (2-$OSO_3$), $R_2 = SO_3^-$, $R_4$ = H, $R_6$ = H (internal standard).

Waters (Milford, MA) protected by a Supelguard LC-18, 2 cm guard column from Supleco (Bellefonte, PA), column oven was maintained at 60 °C. A gradient elution program was run at a flow rate of 1.0 mL/min starting with 96% of eluent A (0.8 mM tetrabutylammonium hydrogen sulfate and 2 mM sodium chloride) and 4% eluent B (acetonitrile) for 3 min to 47% of eluent A, 6% of eluent B and 47% of eluent C (water) in 3.01 min, this condition was maintained for 20 min, followed by a return to initial conditions in 20.1 min. Initial conditions were maintained until 35 min to allow sufficient equilibration time prior to the next injection. The eluate was monitored by a Waters 486 Turnable Absorbance Detector (Milford, MA) iset at 230 nm. The data was acquired and processed with Empower software, Waters (Milford, MA).

### 2.3. Standard solutions

After appropriate serial dilutions working stock solutions of CS-A (100 μg/mL), the three unsaturated disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6S) together (10 and 100 μg/mL) and internal standard ΔDi-UA2S (400 μg/mL) were prepared in water. All the stocks were stored at 4 °C up to 1 month.

### 2.4. Sample preparation

An aliquot of 500 μL plasma was diluted with 400 μL of 50 mM sodium phosphate buffer (pH 7.4) and heated for 3 min at 85 °C in a water bath and cooled in an ice bath. The sample was digested with 50 μL of chondroitinase ABC (50 U/mL) for 6 h at 37 °C. After digestion, 50 μL of internal standard ΔDi-UA2S was added and the sample was centrifuged at 8000 × *g* for 10 min. Five hundred microliters of supernatant was transferred onto a centrifugal filter and centrifuged at 1800 × *g* for 90 min. Ten microliters of clear filtrate was injected onto the HPLC column.

### 2.5. Selectivity

HCP prepared using the above methods was compared against a battery of plasma standards to evaluate the selectivity of the method. This included HCP assayed with the above methods except for chondroitinase ABC treatment, HCP without chondroitinase ABC treatment but externally spiked with disaccharides, HCP fortified with CS-A and treated with chondroitinase ABC.

### 2.6. Calibration curves

Calibration samples were prepared by processing 500 μL of control human plasma in a manner as described above, except instead of digestion with chondroitinase ABC, calibration samples were spiked externally with the appropriate amount of the three unsaturated disaccharides. The calibration samples used had 1, 2, 5, 10 and 20 μg/mL of the three disaccharides. Calibration data was acquired by plotting peak height ratio of each disaccharide and internal standard against the concentration of calibration standards followed by linear regression analysis.

### 2.7. Precision and accuracy

Samples for precision and accuracy were prepared by spiking HCP with the three disaccharides at three different QC levels, i.e., 1.0 μg/mL (lower quality control, LQC; kept same as LLOQ), 5.0 μg/mL (medium quality control, MQC), and 20 μg/mL (upper quality control, UQC), these samples were processed similar to the calibration samples. The intra-day precision and accuracy were estimated by analyzing three replicates containing the three disaccharides at the above three different QC levels. The inter-day precision and accuracy were determined by analyzing the three level QC samples on 3 different days. Accuracy was calculated as percentage deviation (% DEV) from the spiked concentrations and precision was determined as percentage relative standard deviation (% R.S.D.). The criteria for acceptability of the data included accuracy within (±) 15% DEV from the spiked concentrations and a precision within 15% R.S.D. except for LLOQ for which 20% was limit [33]. Inter- and intra-day precision was also determined for measurement of disaccharide representing baseline GAG levels in HCP and HCP fortified with 20 μg/mL of CS-A in triplicates on three different days using this assay.

### 2.8. Auto sampler stability

Stability of disaccharides in the injection solvent was determined periodically by injecting processed LQC, MQC, and UQC samples for up to 48 h in the auto sampler at room temperature, after the initial injection. The peak heights of the analyte obtained in the initial cycle were used as the reference to determine the stability at subsequent points.

### 2.9. Application of the method

The method was used for the determination of endogenous GAGs derived disaccharides in clinical samples obtained from a phase II clinical study of SB-424323. Five hundred milligrams of SB-424323 was orally administered every 12 h on days 1–5 to healthy adult male subjects and healthy surgically sterile or post menopausal females 18–65 years of age. Plasma samples were collected at 6, 2 and 0 h predose on day-1 and 0 h predose on day-5 along with post dose samples at 1, 2, 4, 8, 12, 16, 20 and 24 h on day 5. The plasma was immediately collected and stored at −80 °C until analyzed as described above.

## 3. Results and discussion

Numerous reports have confirmed the presence of GAGs in human plasma [8,17,19,21–24]. In one of the earliest and classical methods which used ten liters of pooled plasma with more than 5 days of multiples steps of purification the detailed distribution of various GAGs in human plasma was described [23]. In this analysis, chondroitin and chondroitin 4-sulfate were identified as major GAGs along with lesser amounts of chondroitin 6-sulfate, hyaluronic acid, heparan sulfate and dermatan sulfate.

Enzyme chondroitinase ABC catalyzes the eliminative cleavage of *N*-acetylhexosaminide linkages in chondroitin sulfate,

chondroitin, dermatan sulfate, and hyaluronic acid, yielding three primary unsaturated disaccharides ΔDi-4S, ΔDi-6S, and ΔDi-0S [34–37]. This indirect method has been used for quantitation of exogenously administered GAG in biological fluids [25–30]. In these methods, GAGs were first removed from the associated proteins via digestion with protease prior to digestion with Chondroitinase ABC. Procedures for removal of associated proteins from GAGs are multistep and time consuming. Chondroitinase AC II have been reported to be used for selective removal of chondroitin sulfate or dermatan sulfate side chains from proteoglycans [38]. In the present method, we found that heat treatment of plasma, followed by incubation with chondroitinase ABC yielded unsaturated disaccharides, which allowed us to obviate the multistep protein removal procedures and increased the throughput.

The released disaccharides were recovered and purified by filtration. The use of filter device was adapted from [28], where microfiltration has been shown to be superior over precipitation methods based on addition of ethanol, methanol or cetylpyridinium chloride to precipitate GAGs after their release from bound proteins which are time consuming and technically difficult [32]. Further, it has been reported that the major GAG in plasma is a small chain undersulfated chondroitin sulfate with molecular mass of 6 kDa [39,40], which cannot be efficiently precipitated by conventional techniques [28].

### 3.1. Specificity and chromatography

The simplicity of the sample cleanup step compromised to some extent the cleanliness of the chromatograms. Peaks corresponding to the three disaccharides and internal standard were identified by comparing their retention times with those obtained from plasma standard and confirmed by spiking technique. Fig. 2(A) and (B) shows typical chromatograms of HCP

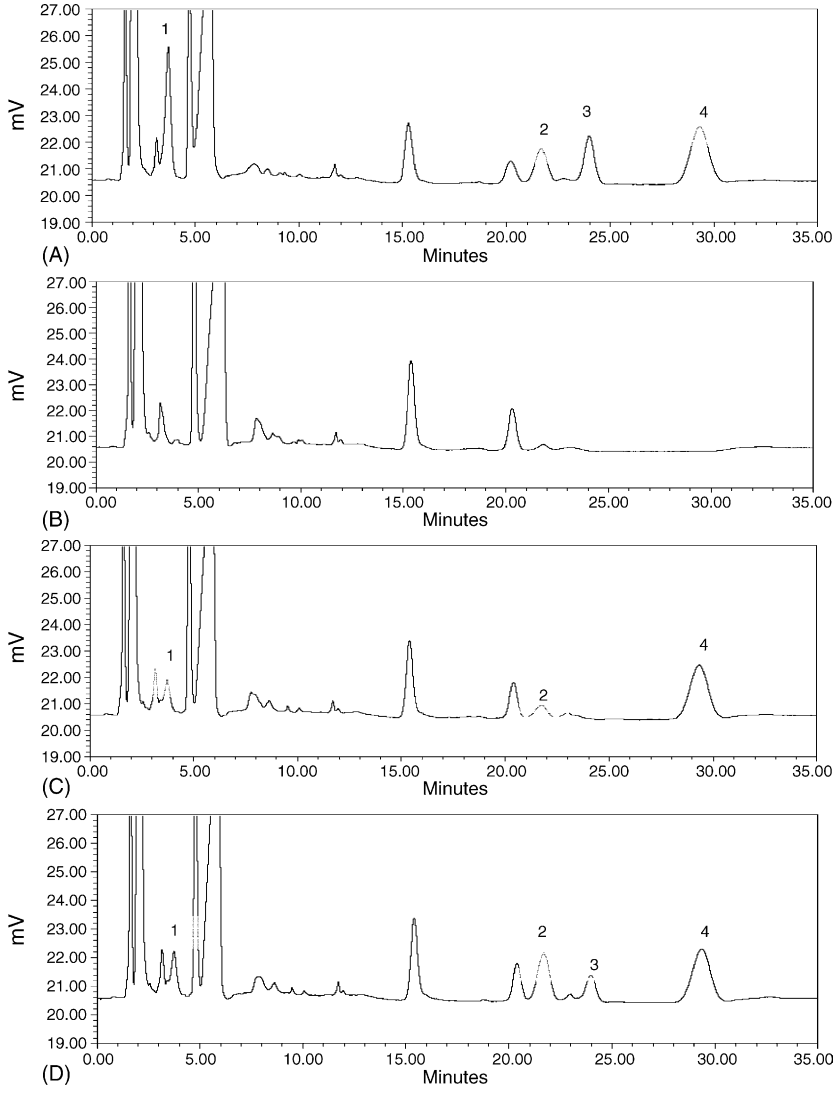


Fig. 2. HPLC chromatogram of a 10 μL injection of (A) human control plasma (HCP) untreated with enzyme chondroitinase ABC and externally spiked with 20 μg/mL each of ΔDi-0S (1), ΔDi-4S (2), ΔDi-6S (3) and 40 μg/mL of internal standard ΔDi-UA2S (4), (B) HCP untreated with enzyme (C) HCP treated with enzyme showing baseline GAGs levels and spiked with 40 μg/mL of internal standard, (D) HCP spiked with 20 μg/mL of CS-A and treated with enzyme and spiked with 40 μg/mL of internal standard. Peaks corresponding to ΔDi-0S, ΔDi-4S, ΔDi-6S and ΔDi-UA2S eluted at 3.7, 21.7, 23.9 and 29.3 min, respectively.

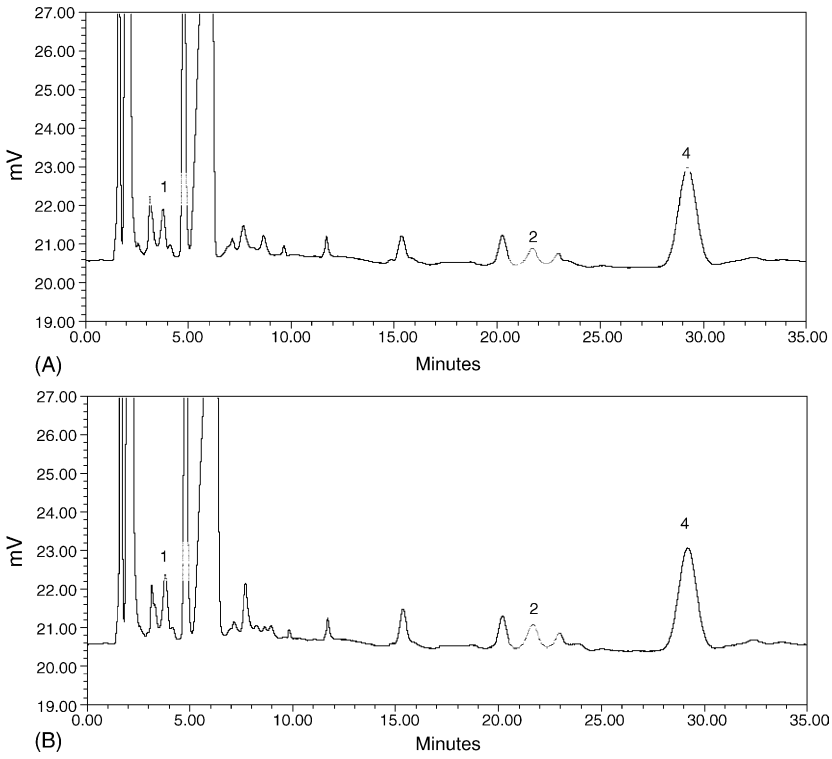


Fig. 3. HPLC chromatogram of a 10 μL injection of plasma sample treated with enzyme chondroitinase ABC from an adult healthy subject (A) day-1 pre-dose and (B) day-5, 4 h post-dose administration of SB-424323, 500 mg every 12 h, days 1–5. Peaks corresponding to ΔDi-0S (1), ΔDi-4S (2), ΔDi-6S (3) and ΔDi-UA2S (4) eluted at 3.7, 21.7, 23.9 and 29.3 min, respectively.

processed with the above method except for the chondroitinase ABC treatment, in (A) HCP was externally spiked with the three disaccharides and internal standard. Nominal retention times for the three unsaturated disaccharides ΔDi-0S, ΔDi-4S, and ΔDi-6S were 3.7, 21.7, and 23.9, respectively, peak corresponding to internal standard ΔDi-UA2S eluted at 29.3 min. Fig. 2(C) and (D) shows typical chromatograms of HCP processed with the above method, in (D) HCP was externally spiked with the CS-A. The disaccharides in (C) represent the baseline GAGs in HCP, fortification of HCP with CS-A resulted in enhancement of the peak heights for disaccharides as seen in (D), this confirmed the disaccharide peaks to be correlated with GAG levels in plasma. Fig. 3, shows representative chromatograms of plasma sample from an adult healthy subject obtained on day-1 pre-dose and day-5, 4 h post-dose administration of SB-424323, 500 mg every 12 h, days 1–5.

### 3.2. Linearity

In the present method, we wished to use more meaningful plasma calibration standards than solution standards for reading concentrations in unknown samples. Baseline GAGs are present in HCP used for the preparation of the calibration standards. Our goal was to measure endogenous GAGs derived disaccharides, hence in the preparation of the calibration samples, we obviated the step of chondroitinase ABC treatment and externally spiked plasma with the known amounts of disaccharides, this allowed use of plasma standards for calibration. Calibration curves for the three disaccharides on 5 separate days were linear ($r^2 > 0.994$) over the concentration in the range of 1.0–20 μg/mL. The LLOQ of the assay was 1 μg/mL.

### 3.3. Accuracy and precision

Accuracy and precision data for determination of each disaccharide in plasma QC samples are presented in Table 1 (intra-day) and Table 2 (inter-day). The with in run (intra-day) accuracy (% DEV) ranged from −16.3 to 16.7, and the between runs (inter-day) % DEV was −8.92 to 5.61. The intra- and inter-day precision (% R.S.D.) in measurement of disaccharides ranged from 0.32 to 20.9, and 3.21 to 18.2, respectively. The bioanalytical method validation guidelines (33), suggest accuracy and precision values to be within ± 15% (20% for LLOQ). Except for at very few instances, the assay conformed to the validation guidelines.

The concentration of disaccharides (mean ± S.D., μg/mL) representing baseline GAGs levels in HCP, measured in triplicates on three different days was 7.96 ± 0.46 and 2.48 ± 0.39 for ΔDi-0S and ΔDi-4S, respectively. Disaccharide ΔDi-6S was found below the LLOQ. Precision in measurement of ΔDi-0S and ΔDi-4S was 5.80 and 15.9% R.S.D., respectively. On spiking HCP with 20 μg/mL of CS-A, the levels of disaccharides (mean ± S.D., μg/mL) ΔDi-0S, ΔDi-4S, and ΔDi-6S were increased to 11.2 ± 1.00, 9.64 ± 1.44, and 5.60 ± 0.64, respectively. Based on these results, the percentage yield of present method in terms of GAGs converting to disaccharides was 80%. Disaccharide ΔDi-0S, ΔDi-4S, and ΔDi-6S were measured with precision 8.93, 14.9, and 11.4% R.S.D., respectively.

Table 1
Intra-day accuracy and precision in determination of unsaturated disaccharides in human plasma

| Spiked concentration (μg/mL) | Run | ΔDi-0S | ΔDi-4S | ΔDi-6S |
|---|---|---|---|---|
| Accuracy (% DEV) | | | | |
| 1.00 (LQC) | 1 | 5.42 | 12.7 | −14.0 |
| | 2 | 1.41 | 7.70 | 2.02 |
| | 3 | −0.60 | −16.3 | −14.7 |
| 5.00 (MQC) | 1 | 12.1 | 16.7 | 14.0 |
| | 2 | 2.79 | −0.34 | 1.32 |
| | 3 | 1.29 | 0.47 | 1.13 |
| 20.0 (UQC) | 1 | 1.94 | −4.92 | −2.54 |
| | 2 | 0.41 | −2.36 | −0.82 |
| | 3 | 6.86 | 5.60 | 6.68 |
| Precision (% R.S.D.) | | | | |
| 1.00 (LQC) | 1 | 3.29 | 20.9 | 5.39 |
| | 2 | 0.61 | 8.62 | 6.39 |
| | 3 | 1.50 | 2.36 | 11.3 |
| 5.00 (MQC) | 1 | 15.5 | 15.8 | 16.5 |
| | 2 | 0.47 | 6.89 | 4.80 |
| | 3 | 2.31 | 5.37 | 5.57 |
| 20.0 (UQC) | 1 | 1.97 | 3.08 | 0.88 |
| | 2 | 0.57 | 0.64 | 0.32 |
| | 3 | 2.27 | 3.45 | 2.23 |

% DEV: % deviation = spiked concentration − measured concentration × 100/spiked concentration; % R.S.D.: % relative standard deviation = S.D. × 100/mean; three replicates at each concentration.

Table 3 summarizes the measurement of disaccharides in HCP along with precision in their determination (Table 4). The precision values for these determinations were within the acceptable limits and this showed that the above method is able to reproducibly, measure the baseline levels of GAGs and any increase in GAG levels indirectly as disaccharides.

### 3.4. Auto sampler stability

To ensure that the disaccharides in the processed samples do not degrade in the auto sampler of the HPLC during analysis. Processed LQC, MQC and UQC samples were injected periodically over a 48 h injection period. The deviations in the concentration as compared with initial injection were found to be less than 15%.

Table 2
Inter-day accuracy and precision in determination of unsaturated disaccharides in human plasma

| Spiked concentration (μg/mL) | ΔDi-0S | ΔDi-4S | ΔDi-6S |
|---|---|---|---|
| Accuracy (% DEV) | | | |
| 1.00 | 2.08 | 1.35 | −8.92 |
| 5.00 | 5.38 | 5.61 | 5.47 |
| 20.0 | 3.07 | −0.56 | 1.10 |
| Precision (% R.S.D.) | | | |
| 1.00 | 3.21 | 18.2 | 11.3 |
| 5.00 | 9.60 | 12.5 | 11.4 |
| 20.0 | 3.23 | 5.34 | 4.38 |

Nine replicates at each concentration.

Table 3
Determination of unsaturated disaccharides (mean ± S.D.) in human plasma representing baseline GAG levels and plasma fortified with CS-A

| Sample | Run | ΔDi-0S | ΔDi-4S | ΔDi-6S |
|---|---|---|---|---|
| Intra-day variation (three replicates) | | | | |
| HCP | 1 | 7.50 ± 0.16 | 2.64 ± 0.20 | BLQ |
| | 2 | 7.86 ± 0.13 | 2.82 ± 0.03 | BLQ |
| | 3 | 8.52 ± 0.10 | 2.00 ± 0.08 | BLQ |
| HCP spiked with 20 μg/mL CS-A | 1 | 10.9 ± 0.12 | 8.46 ± 0.42 | 4.96 ± 0.33 |
| | 2 | 11.5 ± 1.91 | 10.8 ± 1.93 | 6.03 ± 0.74 |
| | 3 | 11.2 ± 0.24 | 9.61 ± 0.38 | 5.82 ± 0.15 |
| Inter-day variation (nine replicates) | | | | |
| HCP | | 7.96 ± 0.46 | 2.48 ± 0.39 | BLQ |
| HCP spiked with 20 μg/mL CS-A | | 11.2 ± 1.0 | 9.64 ± 1.44 | 5.60 ± 0.64 |

BLQ: below limit of quantification; HCP: human control plasma; CS-A: chondroitin sulfate-A.

### 3.5. Clinical Studies

The present validated assay method was used for monitoring plasma GAG levels of 24 healthy adult human subjects in a phase-II clinical study of SB-424323. Fig. 4, shows plasma concentration versus time profiles of individual disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6S) and total (sum of three disaccharides) after oral administration of SB-424323, 500 mg every 12 h, days 1–5 to an adult healthy human subject. The levels of ΔDi-6S were below LLOQ at many time points. The disaccharides concentration in predose samples at 6, 2, and 0 h on day-1, can be considered as representing the true baseline GAGs level. The present method was useful in generating the plasma disaccharide profiles of the three disaccharides representing GAGs levels in healthy adult human subjects enrolled in this phase-II clinical study of SB-424323.

In summary, a simple, accurate and precise method with limited sample processing steps has been developed for determination of endogenous GAGs derived unsaturated disaccharides

Table 4
Precision in determination of unsaturated disaccharides in human plasma representing baseline GAG levels and plasma fortified with CS-A

| Sample | Run | ΔDi-0S | ΔDi-4S | ΔDi-6S |
|---|---|---|---|---|
| Intra-day precision (three replicates) | | | | |
| HCP | 1 | 2.10 | 7.74 | CNC |
| | 2 | 1.60 | 1.17 | CNC |
| | 3 | 1.14 | 4.10 | CNC |
| HCP spiked with 20 μg/mL CS-A | 1 | 1.06 | 4.99 | 6.54 |
| | 2 | 16.7 | 17.8 | 12.2 |
| | 3 | 2.17 | 3.99 | 2.65 |
| Inter-day precision (nine replicates) | | | | |
| HCP | | 5.80 | 15.9 | CNC |
| HCP spiked with 20 μg/mL CS-A | | 8.93 | 14.9 | 11.4 |

CNC: could not be calculated.

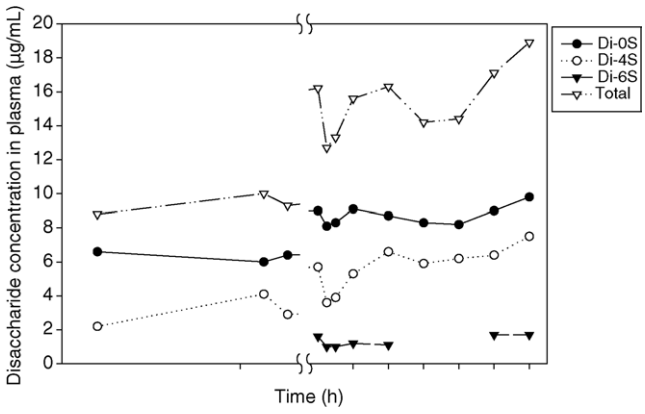


Fig. 4. Plasma concentration versus time profiles of total and individual disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6S) after oral administration of SB-424323, 500 mg every 12 h, days 1–5 to an adult healthy human subject. *X*-axis represents time points, corresponding to plasma samples collected at 6, 2 and 0 h predose on day-1 and 0 h predose on day-5 along with post dose samples at 1, 2, 4, 8, 12, 16, 20 and 24 h on day 5.

in human plasma. This method was successfully used to monitor plasma GAGs derived disaccharide levels in a clinical study of SB-424323.

## References

[1] N.B. Schwartz, L. Galligani, P.L. Ho, A. Dorfman, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 4047.
[2] L. Galligani, J. Hopwood, N.B. Schwartz, A. Dorfman, J. Biol. Chem. 250 (1975) 5400.
[3] F.N. Lugemwa, A.K. Sarkar, J.D. Esko, J. Biol. Chem. 271 (1996) 19159.
[4] K. Takagaki, T. Tazawa, H. Munakata, T. Nakamura, M. Endo, Glycoconj. J. 15 (1998) 483.
[5] P.G. Steg, M. Ziol, O. Tahlil, C. Robert, P. Masson, D. Pruneau, P. Bruneval, P. Belichard, Circ. Res. 77 (1995) 919.
[6] P. Masson, J. Theveniaux, D. Coup, T. Gregoire, M. Vaillot, D. Dupouy, P. Sie, B. Boneu, J. Millet, Thromb. Haemost. 81 (1999) 945.
[7] P.J. Masson, D. Coup, J. Millet, N.L. Brown, J. Biol. Chem. 270 (1995) 2662.
[8] N. Volpi, M. Cusmano, T. Venturelli, Biochim. Biophys. Acta 1243 (1995) 49.
[9] A. Calatroni, R. Vinci, A.M. Ferlazzo, Clin. Chim. Acta 206 (1992) 167.
[10] F. Pasquali, C. Oldani, M. Ruggiero, L. Magnelli, V. Chiarugi, S. Vannucchi, Clin. Chim. Acta 192 (1990) 19.
[11] R.J. Linhardt, T. Toida, Acc. Chem. Res. 37 (2004) 431.
[12] R.L. Jackson, S.J. Busch, A.D. Cardin, Physiol. Rev. 71 (1991) 481.
[13] D.S. Wages, I. Staprans, J. Hambleton, N.M. Bass, L. Corash, Am. J. Hematol. 58 (1998) 285.
[14] D. Carrie, C. Caranobe, A.M. Gabaig, M. Larroche, B. Boneu, Thromb. Haemost. 68 (1992) 637.
[15] H.C. Hemker, S. Beguin, Semin. Thromb. Hemost. 17 (Suppl. 1) (1991) 29.
[16] F.A. Fernandez, M.R. Buchanan, J. Hirsh, J.W. Fenton II, F.A. Ofosu, Thromb. Haemost. 57 (1987) 286.
[17] M. Contini, S. Pacini, L. Ibba-Manneschi, V. Boddi, M. Ruggiero, G. Liguri, M. Gulisano, C. Catini, Br. J. Sports Med. 38 (2004) 134 (discussion 137).
[18] S.O. Kolset, M. Salmivirta, Cell Mol. Life Sci. 56 (1999) 857.
[19] M. Ruggiero, M. Melli, B. Parma, P. Bianchini, S. Vannucchi, Pathophysiol. Haemost. Thromb. 32 (2002) 44.
[20] T.D. Bjornsson, P.V. Nash, R. Schaten, Thromb. Res. 27 (1982) 15.
[21] A. Calatroni, P.V. Donnelly, N. Di Ferrante, J. Clin. Invest. 48 (1969) 332.
[22] I. Staprans, J.M. Felts, J. Clin. Invest. 76 (1985) 1984.
[23] K. Murata, Y. Horiuchi, Clin. Chim. Acta 75 (1977) 59.
[24] S. Cavari, S. Vannucchi, Clin. Chim. Acta 252 (1996) 159.
[25] J. Du, N. Eddington, Anal. Biochem. 306 (2002) 252.
[26] Y. Huang, H. Toyoda, T. Toida, T. Imanari, Biomed. Chromatogr. 9 (1995) 102.
[27] T. Toida, Y. Huang, Y. Washio, T. Maruyama, H. Toyoda, T. Imanari, R.J. Linhardt, Anal. Biochem. 251 (1997) 219.
[28] S. Sakai, J. Onose, H. Nakamura, H. Toyoda, T. Toida, T. Imanari, R.J. Linhardt, Anal. Biochem. 302 (2002) 169.
[29] T. Imanari, T. Toida, I. Koshiishi, H. Toyoda, J. Chromatogr. A 720 (1996) 275.
[30] N. Volpi, Anal. Biochem. 277 (2000) 19.
[31] A. Adebowale, J. Du, Z. Liang, J.L. Leslie, N.D. Eddington, Biopharm. Drug Dispos. 23 (2002) 217.
[32] Y. Emura, T. Mukuda, Seikagaku 45 (1973) 30.
[33] V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanathan, A. Yacobi, Pharm. Res. 17 (2000) 1551.
[34] A. Hamai, N. Hashimoto, H. Mochizuki, F. Kato, Y. Makiguchi, K. Horie, S. Suzuki, J. Biol. Chem. 272 (1997) 9123.
[35] T. Yamagata, H. Saito, O. Habuchi, S. Suzuki, J. Biol. Chem. 243 (1968) 1523.
[36] S. Suzuki, H. Saito, T. Yamagata, K. Anno, N. Seno, Y. Kawai, T. Furuhashi, J. Biol. Chem. 243 (1968) 1543.
[37] H. Saito, T. Yamagata, S. Suzuki, J. Biol. Chem. 243 (1968) 1536.
[38] Y. Oike, K. Kimata, T. Shinomura, S. Suzuki, N. Takahashi, K. Tanabe, J. Biol. Chem. 257 (1982) 9751.
[39] J.J. Enghild, G. Salvesen, S.A. Hefta, I.B. Thogersen, S. Rutherfurd, S.V. Pizzo, J. Biol. Chem. 266 (1991) 747.
[40] H. Toyoda, S. Kobayashi, S. Sakamoto, T. Toida, T. Imanari, Biol. Pharm. Bull. 16 (1993) 945.