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A novel ultra performance liquid chromatography–tandem mass spectrometry method for the determination of sucrose octasulfate in dog plasma

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ABSTRACT

A novel, specific and sensitive bioanalytical method has been developed for the determination of sucrose octasulfate (SOS) in dog plasma and urine using ion-pair reversed-phase ultraperformance liquid chromatography coupled with electrospray triple quadruple mass spectrometry (IPRP-UPLC ESI MS/MS). ^{13}C-labeled sucrose octasulfate-$^{13}C_{12}$ sodium salt is used as the internal standard. 200 μL of plasma or serum sample is extracted using weak anion exchange solid phase cartridge. In this method, a polar amide column is employed for the liquid chromatograph (LC) separation while the diethylamine and formic acid buffer is used as the ion-pairing reagent. The low limitation of quantitation of sucrose octasulfate is 0.20 ng on the column with a signal to noise ratio larger than 50. Parameters such as linearity, accuracy and precision have been validated in full compliance with the FDA guidelines for the bioanalytical method development and validation. A linear regression model fit the calibration curve very well with $R > 0.99$. The bias and coefficient of variation of all levels of QCs are within the range of 15%. The selectivity, matrix effect and stabilities of analytes in solution and matrix have also been evaluated and the results met the acceptance criteria according to the guidelines. Based on these results, the method has qualified to analyze sucrose octasulfate in dog plasma for clinic research. This method has been applied to 1000 preclinical samples.

1. Introduction

Polysulfated carbohydrates are an important class of compounds in terms of biological activity and pharmaceutical importance [1]. For example, heparin has been found to be involved in many biological processes such as blood coagulation, inflammation and even cancer disease [2,3]. Sucrose octasulfate is a synthetic polysulfated carbohydrate. An aluminum salt of sucrose octasulfate (SOS) is clinically used in the treatment of duodenal ulcers. It involves the stabilization of fibroblast growth factor, thus promoting wound healing [4,5]. New and important applications of sucrose octasulfate and its analogs in wound healing and in the treatment of cancer have been suggested from some study [6].

Challenges for the quantitative determination of sucrose octasulfate and its polysulfated carbohydrates family in biological matrix come from the lack of means of sensitive detection. The traditional UV absorption detection is not feasible due to its structure of carbohydrate with which the detection limit cannot meet the requirements of ng/mL, normally larger than 0.5 mg/mL. Fluorescence detection by derivatization usually is a technique to increase the detection sensitivity for HPLC. However, all the polar groups of sucrose octasulfate are sulfate, resulting in unlikely derivatization for fluorescence detection. Furthermore, most of the polysulfated carbohydrates carry many sulfate groups with formally negative charge under nearly all of the experimental conditions, which are labile in the gas phase due to coulomb repulsion. Therefore, their mass spectrum analysis is every challenging. Using mass spectrometer as a detector, the compounds are requested to possess high ionization efficiency and ionized species should be stable in mass spectrometry scale to achieve good sensitivity.

Soft ionization techniques of mass spectrometry have been explored with some success on polysulfated carbonates, such as fast atom bombardment mass spectrometry (FABMS) [7,8], matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) [9–12], and electrospray ionization mass spectrometry (ESI-MS) [13–17]. There is one common problem in these techniques: fragmentation of multiple-negatively charged

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polysulfate carbonates through loss of sulfo group and resulting in low sensitivity. One practical approach for preventing the sulfo loss and consequentially improving sensitivity is to add the counterions to form the stable complex positive ions. An early study of sucrose octasulfate in tetramethylammonium (TMA) matrix proved that stable and predominant $[SOS(TMA)_9]^+$ ion has been formed [7] on FABMS. A systematic approach has been done by Gunay and his coworkers to evaluate the electrospray ionization mass spectral (ESI-MS) analysis of sucrose octasulfate [18] by infusing the solutions. In a survey of ammonium counterions, quaternary ammonium and phosphonium salts were found to give the excellent response in the positive electrospray ionization mode. With cesium ion as counter ion, a sensitive method has been developed for the quantitation of glucose and glycerol [14].

In the present study, a simple, sensitive and rapid LC/MS/MS method for the quantitation of SOS in biological matrix is described. To authors knowledge, this is the first method ever reported for the quantitative analysis of sucrose octasulfate in biological samples using LC–MS/MS. Diethylammonium is capable of forming a cationic adduct with sucrose octasulfate in the positive ESI ionization mode. The stable and predominant doubly charged positive complex ions of sucrose octasulfate can be formed after diethylammonium attachment. The mass spectrometer is operated in MRM mode, and the transitions for analyte and internal standard are monitored. The total runtime is 10 min. This method is highly specific and sensitive, and does not require derivatization or post-column addition. It is ready for clinical research to measure sucrose octasulfate in biological fluids.

2. Material and methods

2.1. Chemicals and materials

Potassium sucrose octasulfate was from USP (Rockville, MD, USA). Phosphoric acid, diethylamine (DEA), formic acid (FA) were from Sigma (Sigma–Aldrich Canada, Oakville, Canada) and sucrose octasulfate-$^{13}C_{12}$ Sodium was custom synthesized. Acetonitrile and methanol were purchased from Fisher Scientific (Fisher Scientific limited, Nepean, Canada). The Strata X-AW 33U Polymeric Weak Anion 30 mg/1 mL cartridges were purchased from Phenomenex (Torrance, CA, USA). Milli-Q water was provided in house by Milli-Pore system (Billerica, MA). Dog plasma (K_2EDTA) was purchased from Biochemed Services (Winchester, VA, USA). All the reagents were used without further purifications.

2.2. LC–MS/MS instrumentation setup

The chromatography is obtained on Waters ACQUITY UPLC system (Waters Canada, Mississauga, Canada) with Waters ACQUITY UPLC column (BEH Amide, 1.7 μm, 2.1 × 100 mm assembled with Waters in-line pre-column filter). Deionized water with 15 mM DEA/FA is used as mobile phase A (MPA) while ACN with 15 mM DEA/FA is used as mobile phase B (MPB). The gradient starts with 1% MPA and increases MPA to 40% over 5 min, then quickly switches MPA to 1% and keeps it to 10 min. Flow rate is 0.40 mL/min and column compartment temperature and sample compartment temperature are maintained at 35 °C and 5 °C, respectively. Under this analytical condition, the retention time of sucrose octasulfate is ∼5.0 min while the retention time of internal standard (IS) (sucrose octasulfate-$^{13}C_{12}$) is ∼5.0 min as well. Mass detector is Waters triple quadrupole (TQD) with ESI positive mode and MRM is employed for monitoring the analyte and internal standard. The analyte MRM transition is 858 → 74 while the IS MRM transition is 864 → 74. Instrument parameters were optimized, such as cone voltage, electrospray voltage, desolvation temperature and desolvation gas to achieve the optimal sensitivity. Milli-Q water was used as strong wash and 80% acetonitrile as weak wash while 10% acetonitrile was used as seal wash.

2.3. Stock preparation, calibration standards and quality controls

About 65.43 mg potassium sucrose octasulfate was weighted into 50 mL of volumetric flask and dissolved with Milli-Q water to make the final concentration of SOS stock solution as 1.0 mg/mL. Internal standard was prepared in the same manner but in much small volume due to the small amount of internal standard obtained. The stock solutions were stored in the refrigerator (5 ± 3 °C) for use or after use. The spiking solutions were diluted from stock solution (1.0 mg/mL) using Milli-Q water to intended concentrations.

The calibration curve range is 50–5000 ng/mL and the spiking solution is not greater than 5% in total of the plasma. The spiked plasma samples of calibration curves with eight calibrants, i.e. STD1 through STD 8, and quality controls (QC), i.e. QC LLOQ, QC Low (QC L), QC Medium (QC M) and QC High (QC H) were stored in −20 °C freezer. Working internal standard of 1000 ng/mL was prepared in deionized water.

2.4. Sample processing

Unknown samples, calibration curve (including blank without internal standard and blank with internal standard) and QC samples are thawed in ice-water bath and vortexed for ∼15 s after completely thawed. A typical extraction procedure is as follows:

- Aliquot 200 μL of plasma sample into 1.5 mL plastic tube and add 100 μL of working internal standard solution except for the blank without internal standard and vortex briefly. Instead, 100 μL of Milli-Q water is added into the blank without internal standard.
- Add 300 μL of 2% phosphoric acid to all samples and vortex briefly to mix well.
- Fix the Strata X-AW 33U Polymeric Weak Anion 30 mg/mL cartridges into the vacuum manifold.
- Add 1.0 mL of methanol followed by 1 mL of water into cartridges and let it drain at gravity flow.
- Load the prepared samples and let them drain slowly under gentle vacuum.
- Wash the cartridges with 1.0 mL of 2% FA in water; let it drain under 2–5 psi vacuum.
- Wash the sample with 1.0 mL of methanol; let it drain fast with vacuum.
- Elute with 0.5 mL of 10:4:100 of DEA:FA:(50/50ACN/H_2O) to the cartridge and let it drain under gentle vacuum.
- Collect the eluent to the tube and transfer 0.3 mL into HPLC insert vial. Inject 10.0 μL into the UPLC–MS/MS system.

2.5. Matrix effect and hemolysis effect

Six individual lots of K_2EDTA dog plasma were spiked at low and high QC level, respectively, i.e., QC L and QC H for matrix effect. Hemolysed plasma was prepared from regular K_2EDTA dog plasma spiked with 2% of whole blood. Three replicates from each level of each lot along with calibration curve were processed and analyzed by following the sample processing procedure described above. Low and high QCs of Hemolysis effect were prepared and processed in the same manner as for matrix effect QCs.

2.6. Stability evaluation

Stabilities in solution and biological matrix were assessed. The area ratio of analyte to internal standard was used to calculate the

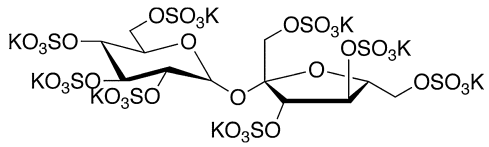

Fig. 1. The structure of potassium sucrose octasulfate.

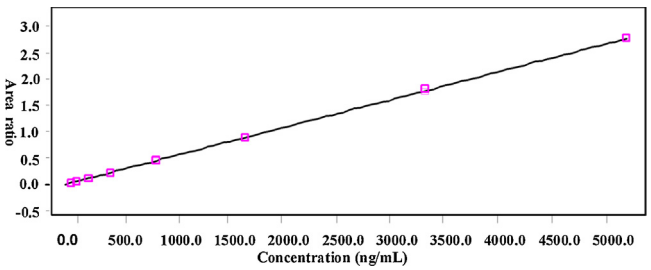

Fig. 3. The calibration curve of SOS in K_2EDTA dog plasma.

difference between aged stock and fresh stock. For the processed sample stability, the processed samples were capped well and put in the refrigerator (5 ± 3 °C) for the intended time. After the intended time, the whole batch (a set of calibration curve and six replicated QCs at each level of QC L and QC H) was injected. The austosampler stability was assessed by comparing the area ratio of fresh samples with that of aged samples, where aged samples (QC L and QC H) were the samples were injected and stored for intended time in autosampler. Three cycles of freeze-thaw stability were evaluated. The stability samples QC L and QC H were stored at −20 °C for at least 24 h in the first cycle and 12 h in the rest of cycles. Everytime, samples were thawed in ice-water bath or fridge in 5 ± 3 °C without any assistance. Six replicates of aged QCs at each level have been tested against the fresh QCs for dog plasma. In addition, the short-term and long-term stabilities of sucrose octasulfate in dog plasma was assessed.

3. Results and discussions

3.1. Detection of sucrose sulfate using mass spectrometry

Sucrose octasulfate is a highly polar compound with eight sulfate groups (Fig. 1). The challenge of effective detection of sucrose octasulfate using mass spectrometry is the formation of the predominant and stable precursor ions of sucrose octasulfate. It is ionic in aqueous solution. A direct electrospray of a sucrose octasulfate water methanol solution does not produce an intensive response in the negative mode. Alternatively, a large amount of negative fragment species of the precursor was observed [18]. This indicates the multiply charged negative precursor ion of sucrose sulfate is fragile in gas phase. The fragility could be caused by two factors: multiple charge of the precursor, which could result in strong coulomb repulsion due to its structure (Fig. 1) and lability of sulfo group in the gas phase. In order to prevent the fragmentation, the formation of complex ion with the attachment of counterions was investigated using tetraalkyl ammonium, cesium, sodium and ammonium and ammine salt [14,18]. The formed positive complex ions are singly charged after nine cations attach to sucrose of octasulfate.

Electrospray ionization is a soft ionization technique. In order to generate the abundant complex ion of sucrose octasulfate, the electrospray technique was employed to exam the above counterions and none of them was found practical for the SOS quantitation on the current LC–MS/MS system due to either the lack of sensitivity or the lack of proper MRM transition. Alternatively diethylamine was found to be a good candidate with the formation of stable and predominant complex ion with two positive charges. In this formed novel complex, hydrogen bonding most likely plays the important role for the stability and the formation of doubly charged complex.

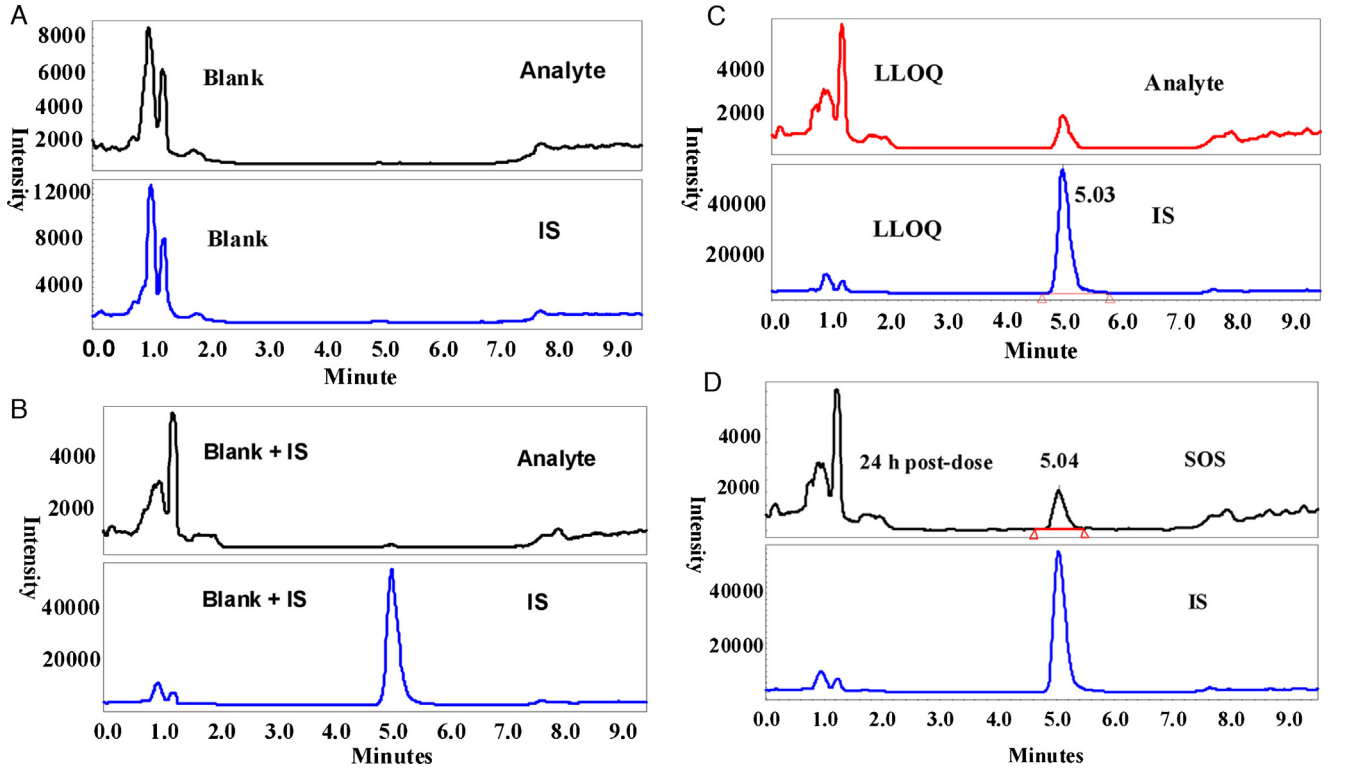

Fig. 2. LC–MS/MS chromatograms of (a) blank dog plasma (b) blank dog plasma with internal standard $^{13}C_{12}$-sucrose octasulfate (c) spiked LLOQ of sucrose octasulfate in dog K_2EDTA plasma and (d) a K_2EDTA dog plasma sample of post-dose after 24 h.

Table 1
Statistic results of the calibration curves spiked in dog plasma (concentration in ng/mL and nominal values are in bracket).

		STD1 (51.92)	STD2 (103.84)	STD3 (207.68)	STD4 (415.37)	STD5 (830.73)	STD6 (1661.47)	STD7 (3322.93)	STD8 (5192.80)	*R*
Day 1	Curve 1	47.80	100.2	206.17	413.5	829.34	1687.69	3393.22	5372.59	0.9981
	Curve 2	59.32	96.46	204.7	399.15	814.71	1681.62	3414.93	5284.21	
Day2	Curve 1	51.07	102.47	204.58	415.66	821.8	1631.49	3368.66	5284.15	1.0000
	Curve 2	53.83	100.6	214.41	412.53	813.28	1651.19	3388.12	5282.79	
Day3	Curve 1	52.38	101.78	208.32	416.45	825.34	1638.33	3389.41	5290.5	0.9991
	Curve 2	54.15	99.86	214.22	411.21	815.98	1635.42	3370.25	5345.35	
Average		53.09	100.23	208.73	411.42	820.08	1654.29	3387.43	5309.93	
CV		7.2%	2.1%	2.2%	1.5%	0.8%	1.5%	0.5%	0.7%	
Average accuracy		102.3%	96.5%	100.5%	99.0%	98.7%	99.6%	101.9%	102.3%	

m/*z* of the formed complex ion is 858, and the MRM transition is 858 → 74. In the complex ion, DEA ammonium ion replaces potassium and a doubly charged ion is formed with the attachment of two extra DEA ammoniums. The internal standard (IS) complex ion is formed in the same manner. The IS MRM transition is 864 → 74.

3.2. Chromatograph

In liquid chromatograph, the addition of diethylamine and formic acid buffer into mobile phase, not only benefits the detection of sucrose octasulfate using mass spectrometer, but also benefits the chromatograph with good peak shape and good retention. On C18 column (data not shown), the complex ion is observed with the elution time around 1.0 min, which could not be adjustable by varying the organic ingredient of mobile phase. This observation indicates that the interaction of the sucrose octasulfate complex with the stationary phase surface of C18 column is weak resulting in the less retention.

However, on an amide column, the SOS complex is eluted around at 5.0 min. The gradient starts with 99% mobile phase B and decreases mobile phase B to 60% over 5 min and then switches to 99%B within 0.1 min. 99% mobile phase B is maintained for 5 min to equilibrate back. With DEA ammonium ions surrounding SOS in the complex, the complex species is very polar, thus the interaction of the complex with the C18 column surface is weak resulting in a less retention of the SOS complex on C18 column. By contrast, on the amide column, the ion pairing reagent (DEA) has much stronger interaction with amide group of the amide column, therefore the complex of SOS can be strongly retained on the amide column. The selected chromatograms are shown in Fig. 2.

3.3. Specificity and selectivity

Shown in Fig. 2 are the LC–MS/MS representative chromatograms of extracted samples: blank dog plasma, blank dog plasma with internal standard and spiked LLOQ in dog plasma. In the dog blank plasma (Fig. 2a), there are no interference peaks observed at the retention times of analyte and internal standard. The addition of internal standard does not bring the interference to analyte (Fig. 2b). Shown in Fig. 2c is the chromatogram of LLOQ (50 ng/mL) with signal to noise greater than 50. This LLOQ is sensitive enough to quantitate SOS in dog plasma (24 h post-dose of SOS) (Fig. 2d). The MRM chromatogram of Fig. 2d also indicates that the present method is highly specific. With 10 μL injection of 50 ng/mL LLOQ, the amount of SOS on column is 0.2 ng (200 μL extraction and elute with 500 μL). In fact, LLOQ at 10 ng/mL can be achieved.

3.4. Linearity, precision and accuracy

The calibration range of 50–5000 ng/mL was assessed for dog plasma. Fig. 3 is a representative graph of calibration curve. With a linear regression model, the bias for all the calibration points is less than 10% while regression coefficient factor *R* is ≥0.995 (Fig. 3). Shown in Table 1 are statistical results of calibration curve of dog plasma from three interday batches. These results demonstrate that the calibration curve in dog plasma possesses a very good linearity.

Table 2
Statistic results of quality controls spiked in dog plasma (concentration in ng/mL and nominal values are in bracket).

	QC LLOQ (51.92)	QC L (162.36)	QC M (1298.85)	QC H (4437.74)
Day 1	49.78	162.89	1334.56	4790.94
	58.42	159.88	1328.14	4813.80
	47.77	160.78	1300.88	4613.48
	52.13	158.42	1315.86	4502.38
	52.36	161.35	1298.45	4687.53
	54.25	166.34	1289.73	4705.42
Day 2	48.36	165.62	1277.48	4546.59
	60.23	156.38	1305.93	4486.67
	52.01	159.87	1301.25	4677.83
	53.48	163.25	1299.47	4509.68
	53.06	160.47	1308.19	4589.48
	55.25	157.59	1315.14	4700.15
Day 3	48.69	157.43	1317.20	4601.58
	60.28	163.21	1288.79	4568.74
	51.08	162.48	1286.73	4756.19
	52.38	158.45	1314.45	4460.38
	55.42	160.11	1297.56	4518.29
	53.81	161.23	1300.45	4638.54
Average	53.26	160.87	1287.08	4490.42
CV	6.9%	1.7%	1.1%	2.4%
Average accuracy	102.6%	99.1%	99.1%	101.2%

Table 3
Matrix effect (concentration in ng/mL and nominal values are in bracket).

	Matrix QC L (162.36)	Matrix QC L accuracy	Matrix QC H (4437.74)	Matrix QC H accuracy
Lot 1	160.09	98.60%	4693.71	105.77%
	148.88	91.70%	4835.08	108.95%
	155.28	95.64%	4765.10	107.38%
Lot 2	174.23	107.31%	4489.32	101.16%
	166.45	102.52%	4788.24	107.90%
	173.42	106.81%	4709.68	106.13%
Lot 3	155.23	95.61%	4556.18	102.67%
	157.38	96.93%	4506.86	101.56%
	160.94	99.13%	4737.18	106.75%
Lot 4	145.78	89.79%	4609.66	103.87%
	160.18	98.66%	4624.35	104.21%
	159.64	98.32%	4600.36	103.66%
Lot 5	168.32	103.67%	4613.85	103.97%
	161.78	99.64%	4801.80	108.20%
	165.24	101.77%	4788.19	107.90%
Lot 6	159.76	98.40%	4612.03	103.93%
	166.21	102.37%	4423.89	99.69%
	164.20	101.13%	4538.69	102.27%
Average	161.28	99.33%	4649.68	104.78%
CV	4.59%		2.60%	
Average accuracy	99.33%		104.78%	

Table 4
Autosampler stability.

Sample Name	Response ratio Fresh injection	Response ratio Injection after 48 h	Sample name	Response ratio Fresh injection	Response ratio Injection after 48 h
QC L 1	0.084	0.094	QC H 1	2.37	2.34
QC L 2	0.082	0.082	QC H 2	2.38	2.37
QC L 3	0.083	0.083	QC H 3	2.36	2.38
QC L 4	0.079	0.081	QC H 4	2.38	2.39
QC L 5	0.084	0.082	QC H 5	2.36	2.38
QC L 6	0.080	0.082	QC H 6	2.37	2.37
Average	0.082	0.084	Average	2.37	2.37
CV	2.4%	5.9%	CV	0.4%	0.8%
Difference%	2.2%		Difference%	0.0%	

QC evaluation results of QC LLOQ, QC L, QC M and QC H are shown in Table 2. Bias at all levels are less than 10% while the precisions of all level are within the range of 10%, well below 15%, which meet the Diteba SOP and the FDA guidelines. These results indicate the current method possesses good reproducibility and accuracy.

3.5. Matrix effect and hemolysis effect

There is no hemolysis effect observed on the analyte. Six individual lots of dog plasma have been examined for matrix effect. Back calculations of matrix QC Ls and QC Hs are presented in Table 3 for dog plasma. Clearly, the results for dog plasma show that there is no significant matrix effect observed on analyte in the this method, where bias of accuracy and precision for both QC L and QC H of 6 individual lots are within 10%.

3.6. Stability

A 15 days of stock solution stability at $5 \pm 3\,^{\circ}C$ was established with an acceptance criteria of ±5.0%. The stability of 96 h has been established for processed samples stored in refrigerator ($5 \pm 3\,^{\circ}C$). The autosampler stability was assessed by comparing the area ratio of fresh samples and aged samples, where aged samples were the samples stored for intended time in autosampler after the fresh samples were injected and then recapped. Table 4 is the results of autosampler stability test with 48 h stored in autosampler. Apparently, the results show that the processed sample after injection in autosampler is stable within 48 h.

Six hours of stability in dog plasma was observed in ice-water bath. Three replicates of each level have been tested for dog plasma. Data in Table 5 shows that there is no obvious degradation for

Table 5
Stability in dog plasma.

Sample name	Response ratio Fresh sample	Response ratio Aged sample (6 h)	Sample name	Response ratio Fresh sample	Response ratio Aged sample (6 h)
QC L 1	0.082	0.083	QC H 1	2.21	2.23
QC L 2	0.084	0.082	QC H 2	2.24	2.22
QC L 3	0.082	0.083	QC H 3	2.21	2.17
Average	0.082	0.082	Average	2.37	2.28
CV	1.48%	0.99%	CV	1.32%	1.53%
Difference%	−0.06%		Difference%	−3.81%	

plasma samples of sucrose octasulfate in ice-water bath for 6 h. Three months of long-term stability under −20 °C storage condition has been established for dog plasma. The stability of freeze-thaw was assessed by three cycles and results met the acceptance criteria of 15%.

4. Summary

The quantitation of SOS in biological fluids is considerably challenging not only because of its detection but also because of its extraction from the matrix. Analytical challenges in the development of an efficient, rugged method for SOS included but were not limited to: detection, LC column selection, peak retention and extraction. The current work has developed a novel rugged and efficient method for the analysis of sucrose octasulfate in Dog plasma at a calibration range of 50–5000 ng/mL. An amide column and ion pairing reagent were used for the separation. The ion pairing reagent in this work benefits not only the retention of polar compounds on the column but also the detection of analytes by mass spectrometer. The precision, accuracy, stability and matrix effect have been assessed. All the results meet the acceptance criteria recommended by FDA and EMA guidelines for the bioanalytical method validation. This method has been used to support the preclinical sample studies and is ready for the test of plasma and urine samples for the clinic research.

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