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Research Article

Method Development and Validation of Short-Chain Brain Sulfatide Using RP-HPLC UV

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A B S T R A C T

The main objective of this research is to present the development and validation of reverse- phase high-performance liquid chromatography RP-HPLC UV method for the determination of short-chain brain sulfatide. The method was performed on HPLC system (Waters, Alliance 2695, USA) equipped with Waters 2487 Dual Absorbance UV/Vis Detector. The column used for the method is Eximius C18 (250mm.L x 4.6mm I.D x 5um) with particle size 5µm and pore size of 100 Å with a guard column from Thermo Fisher Hypersil BDS C18 Guard 10x4mm 5um. The mobile Phase consists of solvent A, 100% methanol and solvent B, methanol: water (1:1, pH 3.5 formic acid) used at a ratio 90:10 (v/v). The flow rate was set at 0.700ml/min and the detection at 254nm. Linearity, precision, accuracy and recovery, specificity and robustness were the parameters validated as per ICH guidelines. The short-chain brain sulfatide's retention time was found to be 3.9 minutes and linearity R2 value was found to be 0.97. The limit of detection (LOD) and the limit of quantification (LOQ) were determined to be115.66 ng/µl and 350.49 ng/µl. The %RSD value for recovery was found to be 2.06. The above findings reveal that this method is simple, precise and accurate method for short-chain brain sulfatide.

KEYWORDS: Brain sulfatide, RP-HPLC, development, validation

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INTRODUCTION

Brain sulfatide (3-O-sulfogalactosylceramide) is a glycosphingolipid, along chain fatty acid abundant in myelin with molecular weight 897.857 (average based on fatty acid distribution in product). The hydrophilic head group is linked to ceramic backbone through beta-glycosidic linkage. The ceramic backbone is a hydrophobic part which contains two hydrocarbon chains. It can be degraded by sulfatase. Sulfatide is involved in B-cell signalling, immune regulation and nervous system development and maintenance. It also plays a crucial role in regulating the differentiation and maturation of oligodendrocyte. Sulfatide is a major component in myelin which helps in organizing and maintaining the structural stability. Brain sulfatides are found predominantly as short chain C16, C18 or long chain C20, C22 or even larger. The ratio of short chain to long chain sulfatides varies by age and also by state of health. Variations in the sulfatide levels can lead to several serious diseases like Alzheimer's disease, Parkinson's disease and Metachromatic Leukodystrophy (MLD). We aimed to develop a method to quantify short chain sulfatide levels in brain samples in compliance with ICH guidelines.

MATERIALS AND METHODS

Instrumentation:

The HPLC system consisted of a HPLC system (Waters, Alliance 2695, USA) equipped with Waters 2487 Dual Absorbance UV/Vis Detector. Data acquisition was through Waters Empower 3.

Reagents and Chemicals:

All reagents were HPLC grade and purchased from Loba Chemie Pvt Ltd, Mumbai, India and Qualigens Thermo Fisher Scientific Pvt Ltd Mumbai, India.

Chromatographic Conditions:

For the separation, the following column were employed, Chromachemie PURITAS Eximius C18 (Catalogue number - CCI-PE18-05-100-250) 250mm.L x 4.6mm I.D x 5um with particle size 5µm and pore size of 100 Å was used.

Preparation of mobile phase:

Mobile phase of solvent A, methanol 100% and solvent B, methanol: water (1:1, pH 3.5 formic acid) was used at a ratio of 90:10. Solvent - Flow rate of 0.700ml was maintained with a run time of 10 minutes around a pressure of 1032 PSI. Column temperatures were maintained at 25°C with an analysis wavelength at 254nm.

Preparation of External Standard Stock Solution:

30 µg of short-chain brain sulfatides, Avanti Research™, Alabama was weighed using weighing balance KingLab – KAB252 Instruments Private Limited, India and dissolved in 90µl of methanol with 10µl of 35% formic acid. 100µl was transferred to amber vials to make stock 1.

1 ml of standard solvent was prepared for dilution.

The solution was sonicated for 5 minutes at 25 °C and transferred to the sample chamber for immediate injection.

Table 1: Dilution Table

Injection No	Dilution	Concentration
1	Stock 1	300 ng/ µl
2	Stock 2	250 ng/ µl
3	Stock 3	200 ng/ µl
4	Stock 4	150 ng/ µl
5	Stock 5	100 ng/ µl
6	Stock 6	50 ng/ µl

Linearity

Linearity is the proportionality between the method's potential to produce a measurable response (detector response) and the concentration of an analyte. The graph was plotted with the concentration of an analyte (on the x-axis) against peak area values (on the y-axis). Six different concentrations of standard stock solutions (50 ng/µl – 300 ng/µl) as mentioned in the table 1 were injected along with the blank. Loading was maintained at 2 µl per injection with the mentioned mobile phase for separation. The regression equation, LOD, LOQ and coefficient of correlation (R2) were determined.

Precision

Precision determines how consistent the results were when the same sample was analysed multiple times. For precision, stock-4 (concentration 150 ng/µl) was injected 3 times. Loading was maintained at 2 µl per injection. The % RSD value of peak area and retention time was determined and analysed.

Accuracy and recovery

The recovery studies were performed at 100%, 150% and 200% and the respective concentrations were 300ng/µl, 225ng/µl and 150ng/µl respectively. The amount of sulfatide recovered at each level was indicated by a peak area. The recovery percentage and RSD values were evaluated.

Specificity

The specificity and selectivity of the method was tested with the blank Plus standard injection and standard alone injection.

Robustness

Robustness was evaluated by varying any parameters. Temperature and flow rate were the two parameters that were considered for validating robustness. The temperature was changed to 27°c and the flow rate was changed to 0.710 ml/min.

RESULTS AND DISCUSSION

Linearity

Six different concentrations (300 ng/µl, 250 ng/µl, 200 ng/µl, 150 ng/µl, 100 ng/µl, 50 ng/µl) were injected into the column. The calibration curve was obtained by plotting concentration and peak area values. The correlation coefficient (R2) was found to be 0.97 which indicates the strong relationship between concentration and response. The LOD and LOQ were found to be 115.66 ng/µl and 350.49 ng/µl respectively.

Table 2 Linearity chart for short-chain brain sulfatide

Concentration ng	Area	
600	5275392	
500	4877891	
400	4757704	
300	4196721	
200	4114951	
100	3720943	
2100	26943602	12830.28667
Total Area divided by Total Volume = Area per Nanogram		**12830.28667**

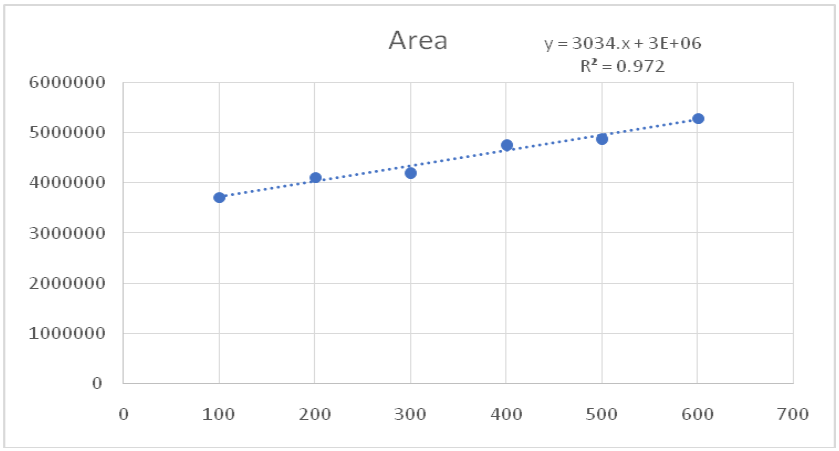

Figure 1: Linearity of Short Chain Brain Sulfatide Graph.

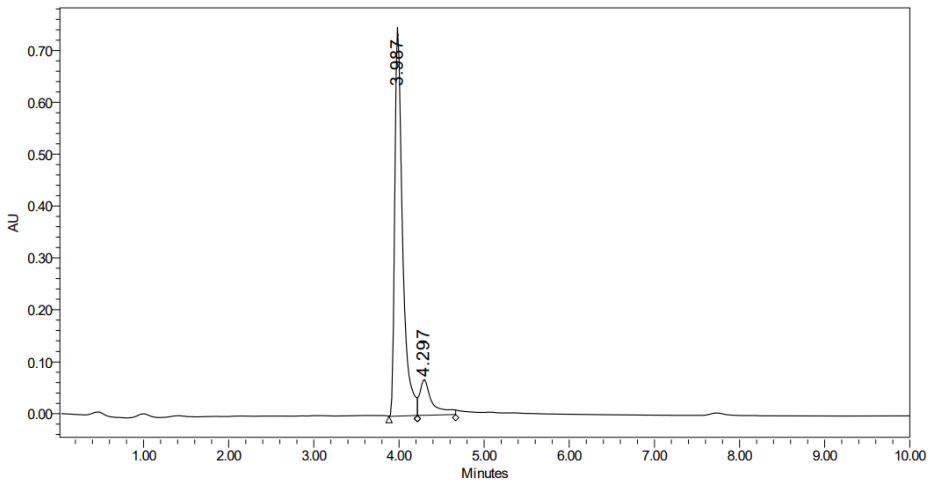

Figure 2: Chromatogram Showing Short Chain Brain Sulfatide with a Retention Time of 3.9

The plotted concentration graph was found to have correlation coefficient (r2) of 0.97. Limits of detection and limits of quantification were found to be at 115.66 ng/µl and 350.49 ng/µl.

Precision

Three replicate injections of concentration (150 ng/µl) were given. The % RSD and peak area was calculated and the % RSD was found to be 0.067 indicating the method's consistency.

Table 3: Precision for short-chain brain sulfatide

Precision		
Sample No	**Concentration**	**Area**
1	150ng/µl	4808719
2	150ng/µl	4283155
3	150ng/µl	4114340
	SD	295687.1879
	Mean	4392358.601
	% RSD	0.067318545

ACCURACY AND RECOVERY

For this accuracy assay, three concentrations (300 ng/µl, 225 ng/µl and 150 ng/µl) were taken at 100%, 75% and 50%. The percentage recovery was found to be 102%, 97% and 99% respectively. The RSD % was found to be 2.06 which demonstrates that the method was accurate

Table 4: Accuracy and Recovery for short-chain brain sulfatide

Accuracy and Recovery				
% Spiked	Area	% Recovered Amount Obtained /Amount	Amount Obtained	Amount Added
100%	3934164	102.2126267	306.63788	300
75%	14024526	97.16481164	1093.104131	1125
50%	1920370	99.7853988	149.6780982	150
	SD	2.061265754		
	Mean	99.7209457		
	% RSD	2.067033901		

Specificity

The Stock 3 (concentration – 100 ng/µl) was injected and evaluated for specificity. The % RSD was found to be 0.014. The absence of non-specific signals in the blank sample demonstrates the method's specificity.

Table 5: Specificity for short-chain brain sulfatide

Specificity	
Sample No	**Area**
Stock 5	4052192
5 µl blank + 45 µl Stock 5	3939085
SD	56553.5
Mean	3995238.256
% RSD	0.014155226

Robustness

By varying flow rate to 0.710 ml/min and temperature to 27°c, there was no significant change observed in the retention time and peak area values. For this assay, Stock 3 (200 ng/µl) concentration was used. The % RSD was found to be 0.033. The above findings state that the analytical method used for brain sulfatide was robust and reliable.

Table 6: Robustness for short-chain brain sulfatide

Robustness	
Parameters	**Area**
Stock 3 - No change in parameter	4860905
change in flow rate to 0.71	4608436
change in temp to 27	4486036
SD	156080.1515
Mean	4649195.579
% RSD	0.033571432

CONCLUSION

This study establishes that the developed method for brain sulfatide using RP-HPLC UV is simple, precise, accurate and effective. The method is validated through linearity, precision, accuracy and recovery, specificity and robustness under the ICH guidelines. The technique's 3.9 retention time showed great linearity, precision, accuracy and recovery, specificity and robustness. The linearity R2 value was found to be 0.97 with the limit of detection of 115.6 and the limit of quantification of 350.49. Furthermore, the method's tolerance to varying experimental conditions increases its suitability for use across different laboratory settings. Applications of this method could be adapted to *in vitro* and *in vivo* studies to quantify brain sulfatide levels.

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