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A Novel LC-ELSD Method for the Quantification of 2-Deoxy D-Glucose Using HILIC Chromatography

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ABSTRACT

A simple and novel method for the direct determination of 2-deoxy D-glucose (2DG) is developed, using a hydrophilic interaction chromatography (HILIC) and evaporative light scattering detection (ELSD). Altech Altima HP HILIC column (250 x 4.6 mm, 5µ particles) was used along with the mobile phase consisted of water and acetonitrile in a linear inverse gradient elution. The proposed method is capable of separating 2DG from its precursor D-Glucal (DGL) and starting material D-Glucose (DGS) with resolution > 3.0. ELSD parameters such as nebuliser temperature and impactor position were found to be critical while detecting DGL. The optimized ELSD parameters were: impactor ON position, nebuliser temperature: 30°C, nitrogen flow rate 1.5 mL/min and gain1. Power regression curves were obtained in the range of 250-750µg/mL with R^2 > 0.999. The percentage recovery of 2DG, DGL and DGS were found to be 99.5±0.7%, 101.1± 2.1, 98.5± 2.2 respectively at 95% confidence interval. The limit of detection (LOD) of DGL and DGS were found to be 20 ng and 15 ng respectively.

Key words: HILIC, LC-ELSD, 2-deoxy D-glucose, D- Glucose, D-Glucal.

INTRODUCTION

2-Deoxy-D-glucose (2DG) is a rare and natural monosaccharide that can be made from D-glucose, D-amino glucose and some amino-polysaccharides [1]. It was first prepared from D-glucose (DGS) in 1922. Chemical structures of 2DG, its precursor D-Glucal (DGL) and its starting material DGS were shown in Fig 1. 2DG is a white crystalline hygroscopic powder. It is odorless, tastes sweet and very soluble in water, partially soluble in hot methanol, ethanol, acetone and butanol.

2DG exhibits various physiological and pharmacological effects on antivirus, anti-cancer and anti-aging [2]. 2DG acts to inhibit the phosphorylation of a glucose molecule that produces glucose-6-phosphate in the glycolysis cycle, therefore inhibiting the production of ATP and can also decrease the temperature in muscle cells. It can restrain viral infections and fermentation, microbes and cancer cell growth. It has great potential in medicine, foods, and cosmetics.

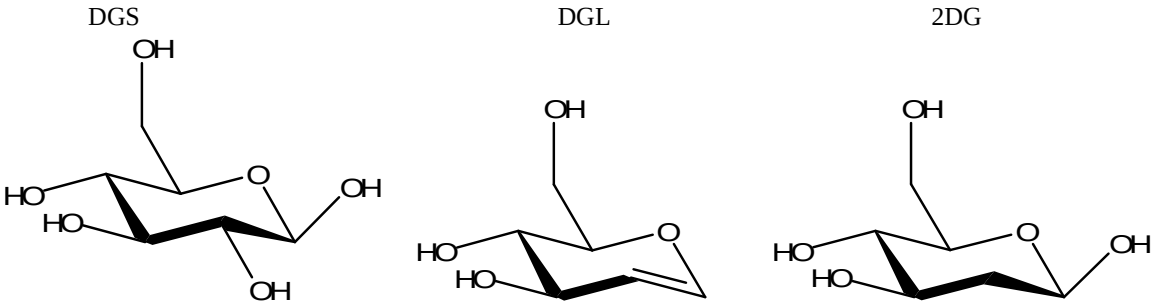

Fig 1. Chemical structures of DGS, DGL and 2DG

A HPLC method was reported in the literature for the determination of Glucosamine, an amino derivative of 2DG in pharmaceutical formulations [3] and also a CE method was reported for the analysis of advanced glycation endproducts formed from the reaction of reducing sugars with the amino group of glucosamine [4]. Threshold Pharmaceutical, Inc., USA, has filed a patent for estimating concentration and purity of crystalline and liquid samples of 2DG by HPLC, which involves an ion exchange column and refractive index (RI) detector [5]. Umegae Y et al, reported simultaneous determination of 2DG & DGS in rat serum by HPLC with post column fluorescence derivatisation [6]. To our knowledge, no HPLC method is available in open literature for the direct determination of 2DG, DGS & DGL in bulk. In this paper, we describe a rapid and sensitive HPLC method for the determination of 2DG, DGS & DGL in bulk using hydrophilic interaction chromatography (HILIC) with evaporative light scattering detector (ELSD).

HILIC is a technique that was designed for the retention and separation of polar compounds. HILIC is a variation of normal phase chromatography and separates compounds by passing a mostly organic mobile phase over a highly polar stationary phase. Analytes elute in order of increasing polarity i.e., the opposite of traditional reversed phase chromatography.

ELSD is increasingly being used in HPLC as a quasi-universal detector eliminating the need for derivatisation of non-absorbing analytes [7]. ELSD operation principle mainly consists of three successive processes: (a) nebulization of chromatographic effluent using an inert gas (e.g. nitrogen), (b) evaporation of mobile phase at relatively low temperature and (c) light scattering by the residual particles, which ideally consist of analytes molecules [8].

MATERIALS AND METHODS

Pure samples of 2DG, DGL and DGS were purchased from Lancaster. Acetonitrile (HPLC grade) was purchased from Qualigens, India. HPLC grade water was obtained from Milli-Q water purification system (Millipore).

Instrumentation

The HPLC system used was Agilent 1100 series HPLC connected with Altech 2000ES model ELSD. Data collection was performed with Chemstation software. Method ruggedness was studied on Waters Alliance 2695 XT with ELSD. Data collection was performed with Waters Empower chromatographic manager.

Preparation of test solutions

The stock solutions of 2DG (5 mg mL^{-1}), DGS and DGL (1 mg mL^{-1}) were prepared separately by dissolving the appropriate amounts of the substances in mobile phase. Necessary dilutions were made to get the test solutions ranging 250-750 µg mL^{-1} for 2DG and 10-50 µg mL^{-1} for DGS and DGL.

Chromatographic conditions

The chromatographic column used was a 250 x 4.6 mm Altima HP HILIC (Altech) with 5µm particles. The chromatographic elution was accomplished with a mobile phase consisting of water and acetonitrile in a linear inverse gradient mode (%ACN): 0-10 min (90-80%), 10-11 min (80-90%), 11-14 min (90%) at a flow rate of 1.0 mL min^{-1}. The column temperature was maintained at 25 ±1°C and the injection volume was 10µL. The chromatographic column effluent was subjected to ELS detection. Nebulization of the effluent in the ELSD was

provided by a stream of nitrogen gas at a flow rate of 1.5mL/min and the nebulized effluent was evaporated at 30°C. The detector gain was set at 1.

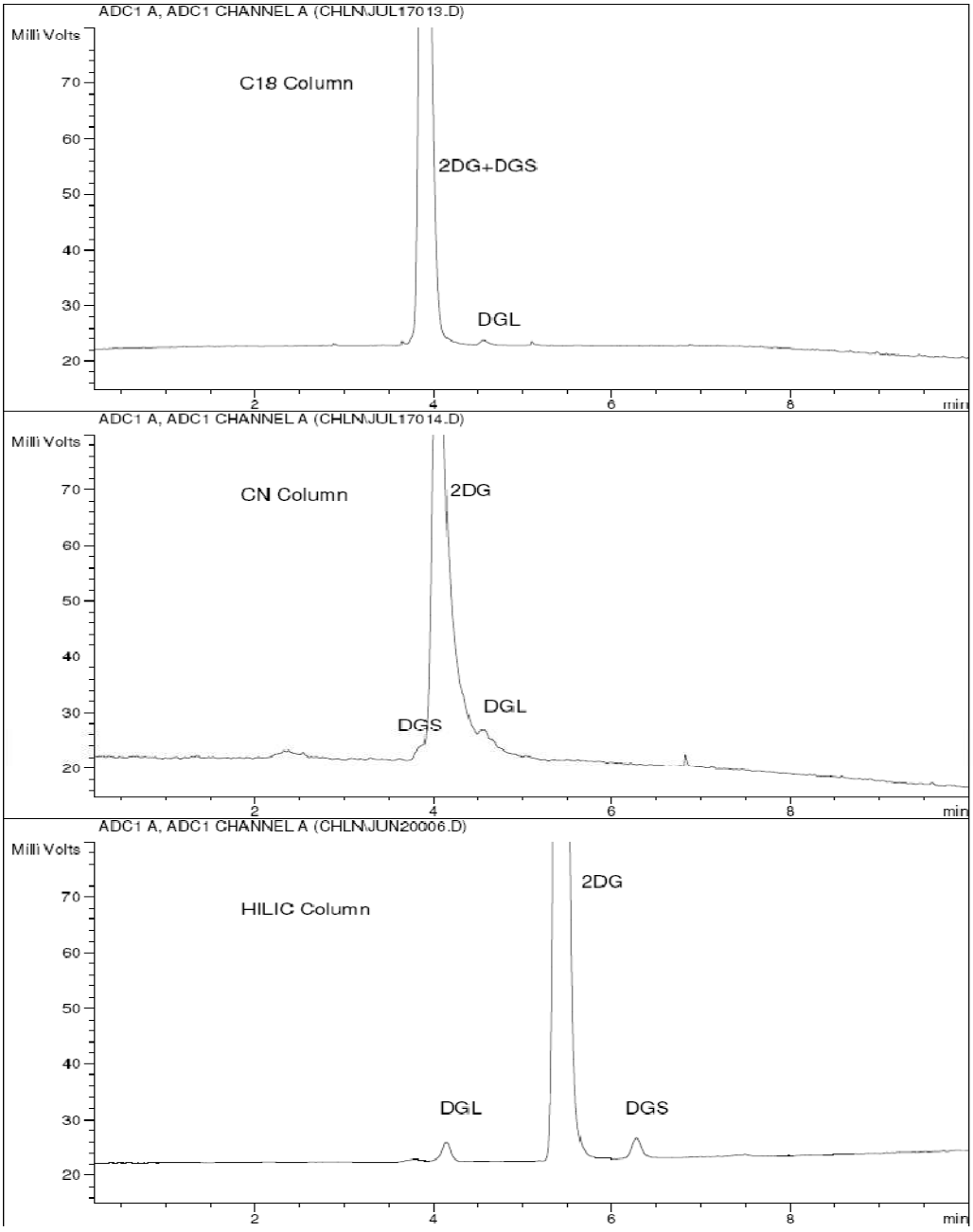

Fig 2. Chromatographic selectivity of DGS, DGL and 2DG using C18, CN and HILIC columns Detector settings

RESULTS AND DISCUSSION

Method development

In order to separate 2DG, DGS and DGL, C18 and CN phases were tried using a mobile phase consisted of water and acetonitrile in a linear gradient elution, but not successful. Further attempts in mobile phase changes using C18 and CN phases, couldn't yield any satisfactory separations. In view of polar nature of the analytes, a HILIC phase was tried. Altima HP HILIC packing is non bonded high purity silica optimized for HILIC separations. Silica

functions as a HILIC packing when small amounts of water are present in the mobile phase. The water creates a layer over the surface of the silica that interacts with polar analytes. In HILIC, a high organic-low aqueous mobile phase is used where the analytes elute in order of increasing hydrophilicity. Increasing the amount of water will elute analytes more quickly. Excellent separation is noticed with Altima HP HILIC column using a mobile phase consisting of water and acetonitrile in a linear but inverse gradient elution. Selectivity chromatograms generated on C18, CN and HILIC phases were shown in Fig. 2. The typical retention times of DGL, 2DG, and DGS were 4.2, 5.5 and 6.2 min. respectively. The chromatographic characteristics were given in Table 1.

Table 1. Chromatographic Characteristics of DGS, DGL and 2DG and method validation data

	DGL	2DG	DGS
Retention time, tR (min)	4.2	5.5	6.2
Theoretical plates, N	9640	10080	13340
Resolution	--	5.5	3.3
Tailing factor	1.3	1.2	1.3
Slope of exponential calibration	0.552	1.326	0.436
Intercept of exponential calibration	4.586	0.713	11.63
Correlation coefficient	0.994	0.9991	0.990
Range, µg mL^{-1}	5-30	250-750	5-30
Precision, RSD (n=6)	2.5	0.36	2.2
Limit of detection, µg mL^{-1}	2	--	1.5
Limit of quantitation, µg mL^{-1}	5	--	4

ELSD can detect all solutes that are less volatile than mobile phase. 2DG and DGS are non volatile compounds, where as DGL is semi volatile and are therefore suitable analytes for ELS detection. Indeed, HILIC chromatography is most favorable condition for ELS detection due to high organic and low aqueous composition of the mobile phases. Two important parameters, nebuliser gas flow rate and drift tube temperature, must be optimized to get best results in ELSD.

The gas flow rate determines the size of droplets formed during nebulization. Higher gas flow rates produce smaller droplets, which evaporate more easily than larger droplets. On the other hand, smaller droplets are less effective in light scattering and therefore produce smaller signals than larger droplets. Different gas flow rates ranging 1.5-2.5 mL min^{-1} were checked and the flow rate at 1.5 mL min^{-1} was found to be the optimum (Fig 3).

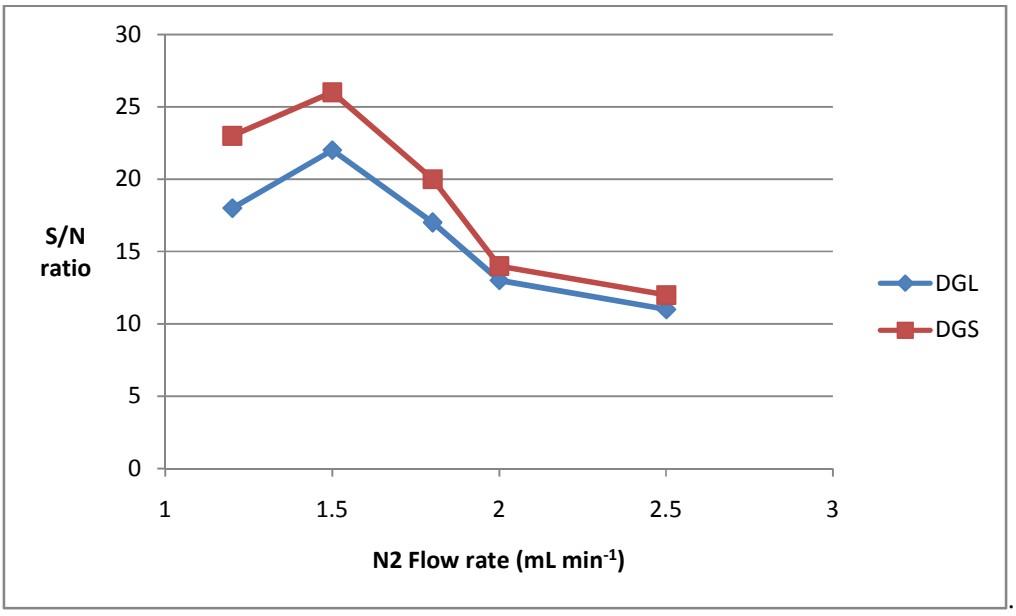

Fig 3. Effect of nebuliser gas flow rate on S/N ratio

The proper drift tube temperature setting will be based on mobile phase volatility, mobile phase flow rate, nebuliser gas flow rate and analyte volatility. Owing to the semi volatile nature of DGL, different drift tube temperatures ranging 30-55 °C were studied and 30°C was found to be the optimum (Fig 4).

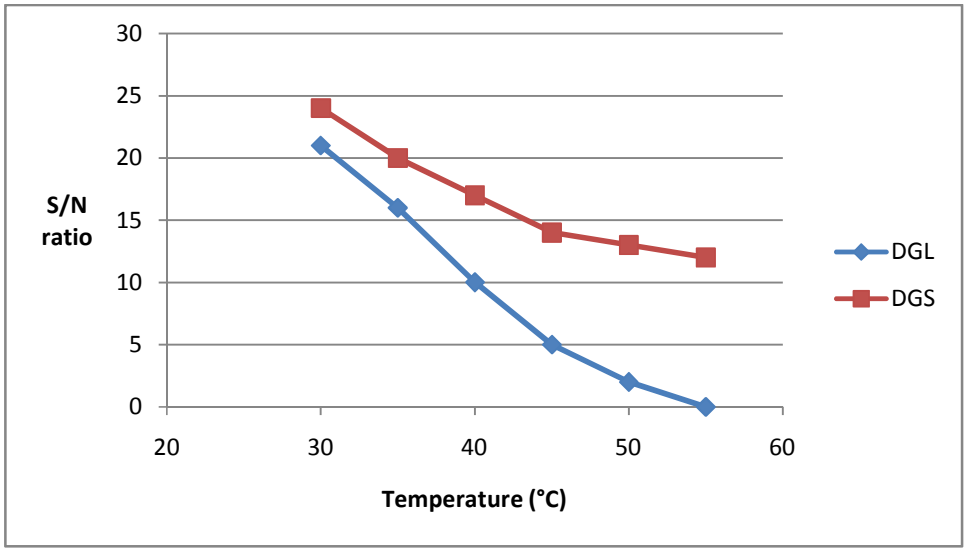

Fig 4. Effect of temperature on S/N ratio

Two different impactor positions "off" and "on" were possible with Altech 2000 ES detector. Position "off" (non-volatile compounds) allows maximum sensitivity by allowing the entire sample stream to reach the optical cell. With impactor position "on" (semi volatile compounds), a portion of the sample stream is diverted to a drain tube. As illustrated in Fig 5, it was proved that the optimal impactor setting was "on". DGL was not detected in Impactor "off" position due to its semi volatile nature.

Method Validation

The validation was done according to ICH guidelines [9]. The target analyte concentration of 2DG was fixed as 500 µg mL^{-1}. Specificity of the method was demonstrated by separating DGS and DGL from 2DG. The system precision was assessed by multiple injections of standard solution of 2DG at target analyte concentration. For six injections of the solution, the relative standard deviation (RSD) was 0.4%. Linearity of the method was determined by preparing and analyzing five standard solutions in the range of 250-750 µg mL^{-1}. The peak areas (Y) were correlated to the analyte mass (x) by the well established power regression curve of ELSD response [8]

$Y = axb \Rightarrow \log Y = b\log x + \log a$

where a and b are coefficients depending on instrumental parameters, nature and concentration of analyte, gas and liquid flow rates, evaporation temperatures etc.

Power regression analysis of peak areas versus concentration data yielded a $R^2 > 0.999$ for 2DG. Regression line equation was y = 0.713x1.326. Accuracy of the method was determined by analyzing 2DG sample at three different concentrations levels 400, 500 and 600 µg mL^{-1}. The percentage recovery of 2DG was found to be 99.5±0.7% at 95% confidence interval (Table 2). Method ruggedness was studied using different make of instruments specified in Experimental section. Typical chromatogram generated in ruggedness study is shown in Fig 6.

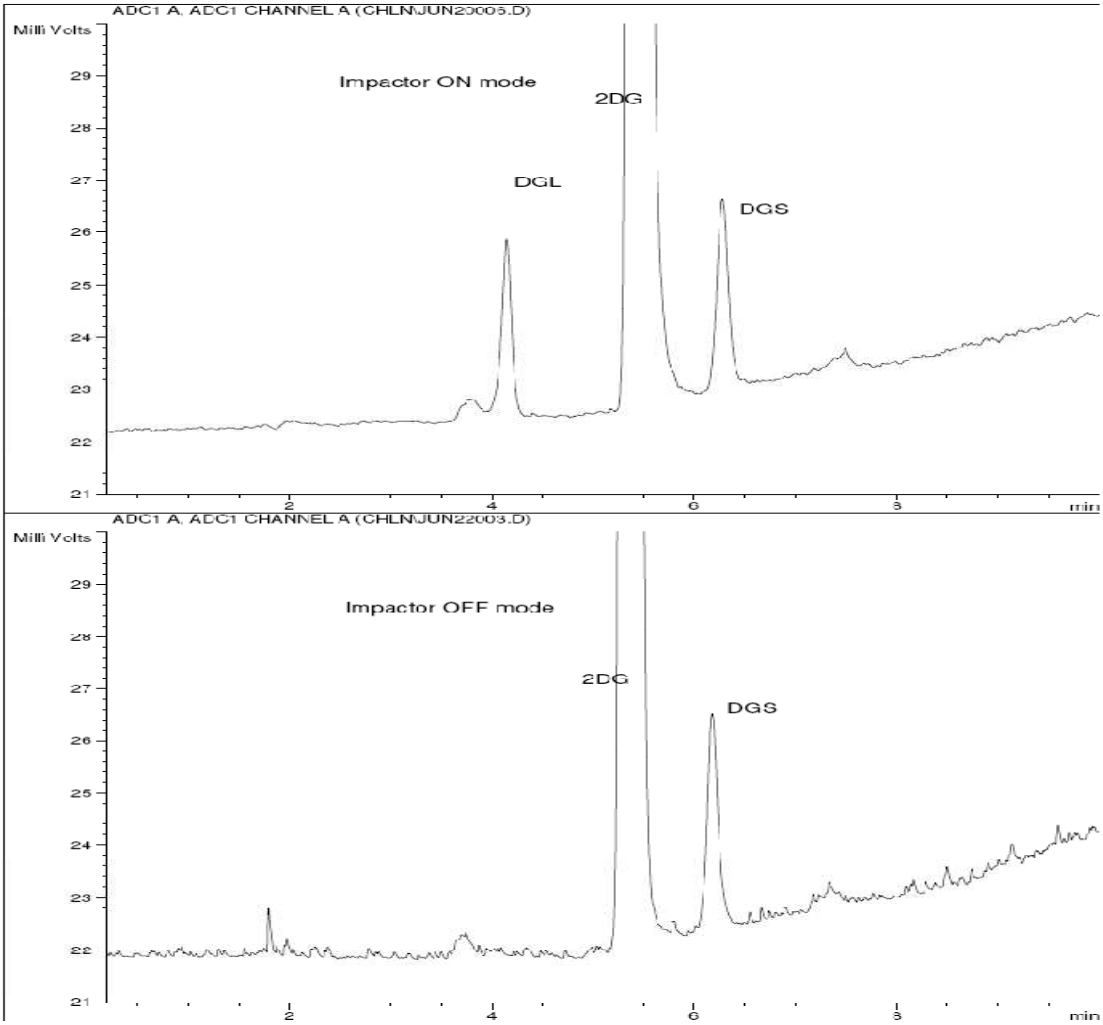

Fig 5. Effect of impactor position on DGL detection

Table 2. Accuracy in the assay determination of 2DG

Taken (µg)	Recovered (µg)	% Recovery	% RSD
400.4	394.6	98.6	0.2
	395.8	98.9	
	394.4	98.5	
501	503.4	100.5	0.2
	503.2	100.4	
	501.6	100.1	
600.2	595.6	99.2	0.7
	595.2	99.2	
	603	100.5	

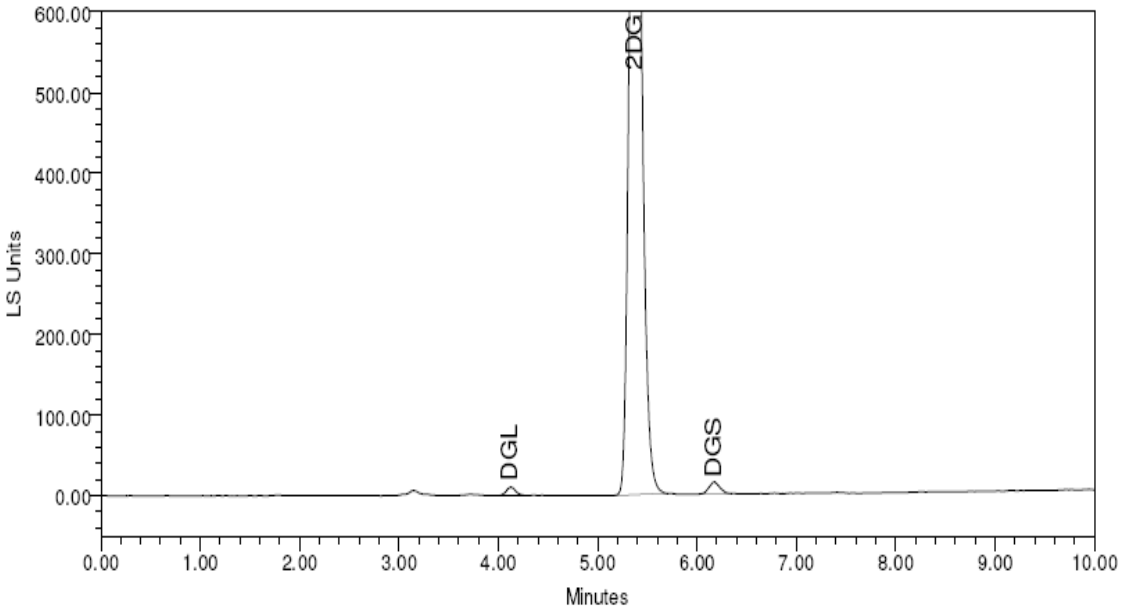

Fig 6. Typical selectivity chromatogram generated during ruggedness study

Quantification of DGL and DGS

Standard addition and recovery experiments were conducted in the presence of 2DG to determine accuracy of the present method for the quantification of DGL and DGS. The study was carried out at 10, 15 and 20 µg mL^{-1}. The recovery of DGL and DGS were calculated from the calibration curve drawn in the concentration range of 5-30 µg mL^{-1}. The calibration equation for DGL and DGS were found to be y = 4.586x0.552 and y = 11.63x0.436 respectively. The percentage recovery of DGL and DGS were found to be 101.1± 2.1 and 98.5± 2.2 respectively at 95% confidence interval (Table 3).

Table 3. Recovery results of DGL and DGS

Added (µg)	Recovered (µg)	% Recovery	% RSD (n=3)
DGL			
10.1	10.47	103.7	3.5
14.9	15.11	101.4	2.8
20.3	19.9	98	2.5
DGS			
9.8	9.46	96.5	3.1
14.8	14.37	97.1	2.9
20.1	20.5	102	2.3

n, Number of determinations

The limit of detection (LOD) of DGL and DGS were 2 and 1.5 µg mL^{-1} respectively for 10µL injection volume. The limit of quantitation (LOQ) of DGL and DGS were 5 and 4 µg mL^{-1} respectively for 10µL injection volume.

CONCLUSION

A simple HPLC/ELSD method using HILIC column was described for the direct determination of 2-DG. This method was able to separate the starting material DGS and precursor DGL from 2-DG. Gas flow rate, drift tube temperature and impactor position were found to be critical while optimizing ELSD response. Correlation between ELSD response and analyte concentration was checked using power regression analysis and the accuracy of the method was found to be good. Furthermore, this method is useful for the quantification of DGL and DGS. However, the LOQ of DGL and DGS were limited to 1% and 0.8% of the product concentration respectively.

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