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Research Article

An Rp-Hplc Method Development and Validation for the Estimation of Dasiglucagon in Bulk

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Abstract

A simple, Precise, Accurate method was developed for the estimation of Dasiglucagon by RP-HPLC technique. Chromatographic conditions used are stationary phase Ascentis C18 (150mm*4.6mm3.6m), Mobile phase 0.01N potassium dihydrogen phosphate: Acenotrile in the ratio of 60:40 and flow rate were maintained at 0.6ml/min, detection wave length was 230nm, column temperature was set to 30oC and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. Linearity study was carried out between 25% to150 % levels, R2 value was found to be as 0.999. Precision was found to be 0.9 for repeatability and 0.8 for intermediate precision. LOD and LOQ are 0.027mg/ml and 0.082mg/ml respectively. By using above method assay of marketed formulation was carried out 100.10% was present. Degradation studies of Dasiglucagon were done, in all condition's purity threshold was more than purity angle and within the acceptable range.

Keywords: HPLC Dasiglucagon, Method development, ICH Guidelines

Introduction

Dasiglucagon is the first glucagon medicine available in an aqueous formulation. It has a free NH3 group at the N-terminus and a COOH group at the C-terminus and is separated as a hydrochloride salt [1]. Dasiglucagon, like natural glucagon, has 29 amino acids, but with 7 amino acid changes intended to improve both physical and chemical stability in aqueous solutions [2]. Dasiglucagon, unlike natural glucagon, has a substantially decreased tendency to clump in aqueous solutions [3]. Severe hypoglycaemia, a frequent and serious adverse effect of insulin therapy, necessitates prompt medical attention [4]. Hypoglycaemia requires rapid treatment. For many people, a fasting blood sugar of 70 mg/dL, or 3.9 mmol/L, or below should act as a warning for hypoglycaemia [5]. Few analytical techniques, such as potentiometric titration HPTLC [6], RP-HPLC [7], HPLC [8], LC [9] and UV Spectrophotometry [10], are available, according to literature survey. The primary purpose of this research is to develop an RP-HPLC [11] method that is rapid, simple, and ac curate for determining the amount and type of Dasiglucagon medications [12]. As indicated by the ICH, a tried-and-true method was employed to estimate the results (Figure 1).

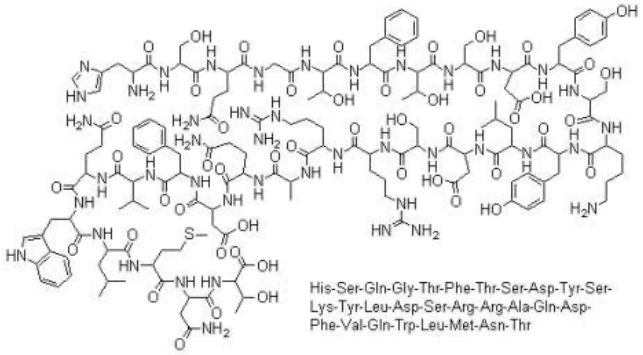

Figure 1: Structure Of Dasiglucagon.

Materials and Reagents

Dasiglucagon Pure Drugs (API), Combination Dasiglucagon tablets (ZEGALOGUE), Distilled water, Acetonitrile, Phosphate buffer, Methanol, Potassium dihydrogen ortho phosphate buffer, Ortho-phosphoric acid. All the above chemicals and solvents are from Rankem.

Instrumentation and Chromatographic Conditions

HPLC instrument used was of WATERS HPLC 2965 SYSTEM with Auto Injector and PDA Detector. Software used is Empower 2. UV-VIS spectrophotometer PG Instruments T60 with special bandwidth of 2mm and 10mm and matched quartz was be used for measuring absorbance for Dasiglucagon solutions. Sonicator (Ultrasonic sonicator) PH meter (Thermo scientific) Micro balance (Sartorius) and Vacuum filter pump.

Reagents Used

Methanol HPLC Grade (RANKEM), Acetonitrile HPLC Grade (RANKEM), HPLC grade Water (RANKEM). Based on drug solubility and P^{ka} Value following conditions has been used to develop the method estimation of Dasiglucagon.

Method Development

Trial 1

Chromatographic Conditions

Column : Ascentis 150mm x 4.6 mm, 5.0m.

Mobile phase : Water: Methanol (50:50)

Flow rate : 1.0 ml/min

Detector : PDA 230nm

Temperature : 300C

Injection Volume: 10mL

(Figure 2).

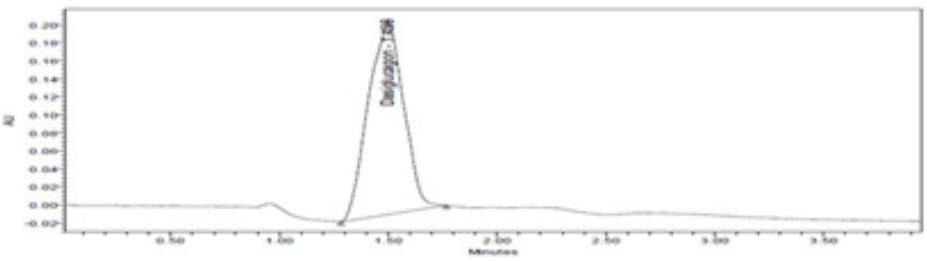

Figure 2: Trial Chromatogram -1.

Observation: Peak shape was not good and retention time was less, and USP Plate count was not good so further trial was carried out.

Trial 2

Chromatographic Conditions

Column : Ascentis C18 150mm x 4.6 mm, 5.0m.

Mobile phase : 50% Acetonitrile: 50% Orthophosphoric acid

Flow rate : 1.0 ml/min

Detector : PDA 230nm

Temperature : 30˚C

Injection Volume: 10mL

(Figure 3).

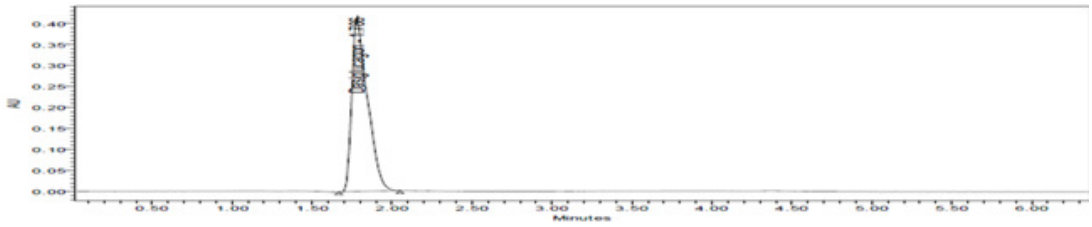

Figure 3: Trial Chromatogram -2.

Observation: In this trail peak was elute with good peak shape and all system suitability parameters are within the limit, but analyte peak was very less. So, further trail was carried out.

Optimized Method

Optimized Chromatographic Conditions

Column : Ascentis C18 150mm x 4.6 mm, 5.0m.

Mobile phase : 0.01N potassium dihydrogen phosphate: Acenotrile (60:40)

Flow rate : 0.6 ml/min

Detector : PDA 230nm

Temperature : 30˚C

Injection Volume: 10mL

(Figure 4).

Observation: All the system suitability parameters were within the range and satisfactory as per ICH guidelines.

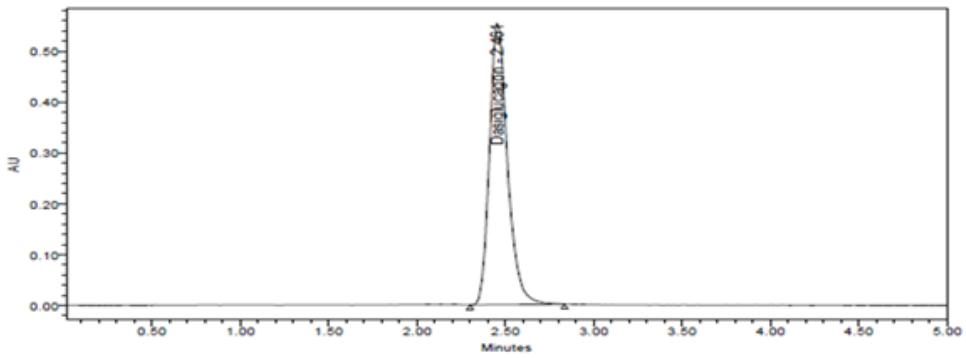

Figure 4: Optimized Chromatogram -3.

Diluent

Based up on the solubility of the drugs, diluent was selected, Acetonitrile and buffer taken in the ratio of 50:50

a) **Preparation of Standard stock solutions:** Accurately weighed 12mg of Dasiglucagon transferred 100ml and volumetric flasks, 3/4th of diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labelled as Standard stock solution (120mg/ml of Dasiglucagon).

b) **Preparation of Standard working solutions (100% solution):** 1ml of Dasiglucagon from each stock solution was pipetted out and taken into a 10ml volumetric flask and made up with diluent. (12mg/ml of Dasiglucagon).

c) **Preparation of Sample stock solutions:** Transfer 2 single dose vails into a 10 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min; further the volume was made up with diluent and filtered by HPLC filters (120 mg/ml of Dasiglucagon).

d) **Preparation of Sample working solutions (100% solution):** 1ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (12mg/ml of Dasiglucagon)

e) **Preparation of buffer:** Buffer: 0.01N KH_2PO_4 Buffer: Accurately weighed 1.36gm of Potassium dihydrogen Ortho phosphate in a 1000ml of Volumetric flask add about 900ml of milli-Q water added and degas to sonicate and finally make up the volume with water then PH adjusted to 4.8 with dil. Orthophosphoric acid solution.

0.1% OPA Buffer: 1ml of ortho phosphoric acid was diluted to 1000ml with HPLC grade water. Take 600ml of 0.01N KH_2PO_4 Buffer and 400ml acetonitrile added and degas to sonicate it for 10min.

Validation

System Suitability Parameters

The system suitability parameters were determined by preparing standard solutions of Dasiglucagon (12ppm) and the solutions were injected six times and the parameters like peak tailing, resolution and USP plate count were determined. The % RSD for the area of six standard injections results should not be more than 2%.

a) **Specificity:** Checking of the interference in the optimized method. We should not find interfering peaks in blank and placebo at retention times of these drugs in this method. So, this method was said to be specific.

b) **Precision:** Preparation of Sample stock solutions: Transfer 2 single dose vails into a 10 ml volumetric flask, 5ml of diluents was added and sonicated for 25 min, further the volume was made up with diluent and filtered by HPLC filters (120 mg/ml of Dasiglucagon and filtered by HPLC filters. (120mg/ml of Dasiglucagon)

c) **Preparation of Sample working solutions (100% solution):** 5ml of filtered sample stock solution was transferred to 10ml volumetric flask and made up with diluent. (12mg/ml of Dasiglucagon)

Linearity

Preparation of Standard stock solutions: Accurately weighed 12mg of Dasiglucagon transferred 100ml and volumetric flasks, 3/4th of diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labelled as Standard stock solution (120mg/ml of Dasiglucagon).

a) **25% Standard solution:** 0.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (3mg/ml of Dasiglucagon)

b) **50% Standard solution:** 0.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (6mg/ml of Dasiglucagon)

c) **75% Standard solution:** 0.75ml each from two standard stock solutions was pipetted out and made up to 10ml. (9mg/ml of Dasiglucagon)

d) **100% Standard solution:** 1.0ml each from two standard stock solutions was pipetted out and made up to 10ml. (12mg/ml of Dasiglucagon)

e) **125% Standard solution:** 1.25ml each from two standard stock solutions was pipetted out and made up to 10ml. (15mg/ml of Dasiglucagon

f) **150% Standard solution:** 1.5ml each from two standard stock solutions was pipetted out and made up to 10ml. (18mg/ml of Dasiglucagon).

Accuracy

a) **Preparation of Standard stock solutions:** Accurately weighed 12mg of Dasiglucagon transferred 100ml and volumetric flasks, 3/4th of diluents was added and sonicated for 10 minutes. Flasks were made up with diluents and labeled as Standard stock solution (120mg/ml of Dasiglucagon).

b) **Preparation of 50% Spiked Solution:** 0.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

c) **Preparation of 100% Spiked Solution:** 1.0ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

d) **Preparation of 150% Spiked Solution:** 1.5ml of sample stock solution was taken into a 10ml volumetric flask, to that 1.0ml from each standard stock solution was pipetted out, and made up to the mark with diluent.

e) **Acceptance Criteria:** The % Recovery for each level should be between 98.0 to 102.

Robustness

Small deliberate changes in method like Flow rate, mobile phase ratio, and temperature are made but there was no recognized change in the result and are within range as per ICH Guide lines. Robustness conditions like Flow minus (0.9ml/min), Flow plus (1.1ml/min), mobile phase minus, mobile phase plus, temperature minus (25°C) and temperature plus (35°C) was maintained and samples were injected in duplicate manner. System suitability parameters were not much affected and all the parameters were passed. %RSD was within the limit.

LOD Sample Preparation

0.25ml of Standard stock solution was pipetted out and transferred to 10ml volumetric flasks and made up with diluents. From the above solution 0.1ml Dasiglucagon, were transferred to 10ml volumetric flasks and made up with the same diluents.

LOQ Sample Preparation

0.25ml of Standard stock solution was pipetted out and transferred to 10ml volumetric flasks and made up with diluents. From the above solution 0.3ml Dasiglucagon, were transferred to 10ml volumetric flasks and made up with the same diluents.

Assay Methodology

Assay of the marketed formulation was carried out by injecting sample corresponding to equivalent weight into HPLC system. And percent purity was found out by following formulae.

Calculate the percentage purity of Dasiglucagon present in tablet using the formula:

Calculation:

$$\text{Assay} = \frac{\text{Spl area}}{\text{Std area}} \times \frac{\text{Std. Dil. Fac}}{\text{Spl. Dil. Fac}} \times \frac{\text{Avg. Wt. of Tab}}{\text{L.C}} \times \text{Potency of Std}$$

Spl area - Sample Peak area

Std area – Standard Peak area

Std. Dil. Fac- standard dilution factor

Spl. Dil. Fac- sample dilution factor

Avg. Wt. of Tab- average weight of tablet

L.C - lable claim

Potency of Std

Results and Discussions

System Suitability

A Standard solution of Dasiglucagon working standard was prepared as per procedure and was injected five times into the HPLC system. The system suitability parameters were evaluated from standard Chromatograms obtained by calculating the % RSD of retention time, tailing factor, theoretical plates and peak areas from five replicate injections are within range and Results were shown in table 6.1. (Table 1, Figures 5,6).

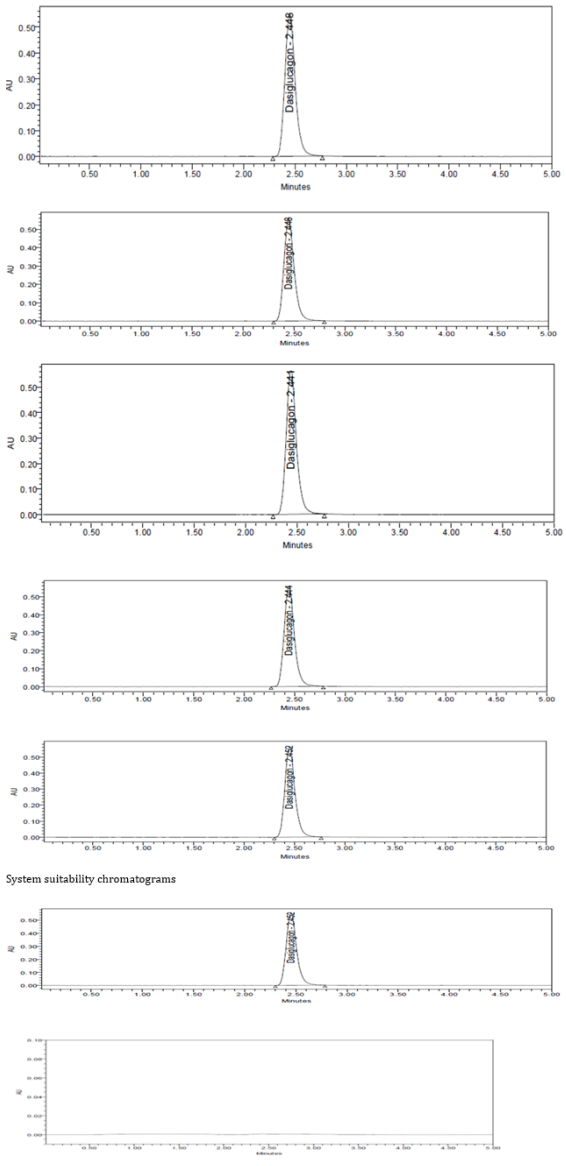

Figure 5: Blank Chromatogram.

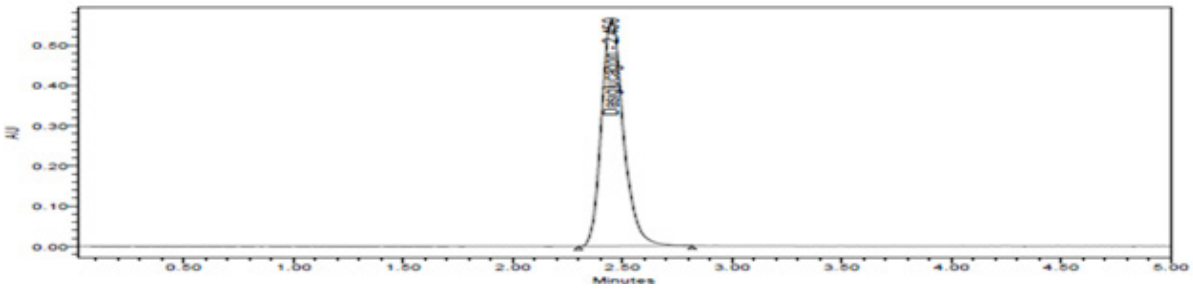

Figure 6: System suitability Chromatogram.

Table 1: System Suitability Parameters.

Peak Name: Dasiglucagon

	Peak Name	RT	Area	USP Plate Count	USP Tailing
1	Dasiglucagon	2.441	3900986	3107	1.2
2	Dasiglucagon	2.444	3940420	2867	1.2
3	Dasiglucagon	2.448	3895207	2771	1.23
4	Dasiglucagon	2.448	3855454	3036	1.21
5	Dasiglucagon	2.452	3922067	3402	1.21
6	Dasiglucagon	2.452	3923727	3126	1.23
Mean			3906310		
Std. Dev.			29843.3		
% RSD			0.8		

Precision

Repeatability: Six working sample solutions of 12ppm are injected and the % Amount found was calculated and %RSD was found to be 0.9 and chromatogram was shown in figure (Table 2, Figure 7).

Table 2: Repeatability data.

S. No	Peak Area
1	3950268
2	3908540
3	3899032
4	3895431
5	3984869
6	3918533
AVG	3926112
STDEV	34861.9
%RSD	0.9

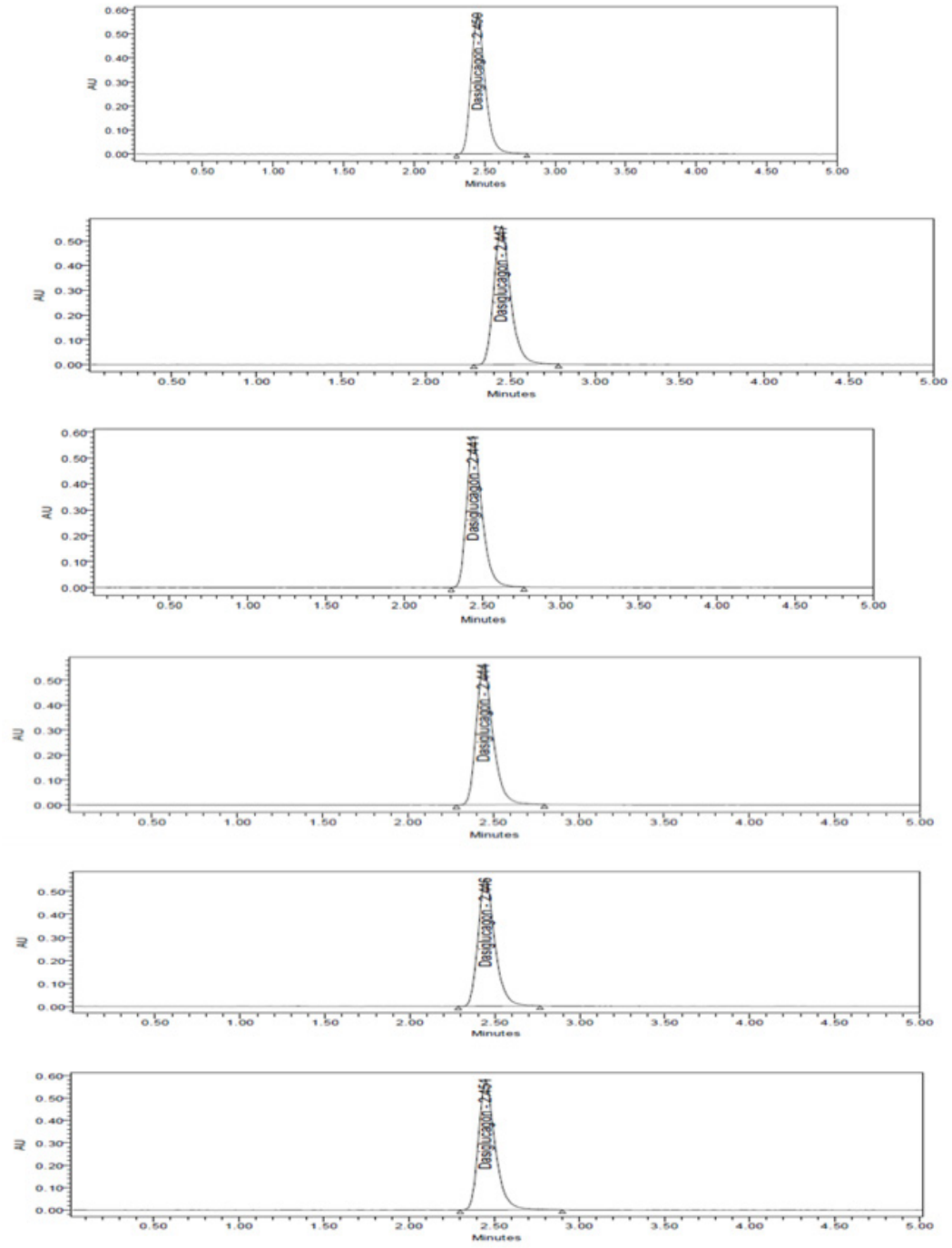

Figure 7: Repeatability Chromatogram.

Intermediate precision: Six working sample solutions of 12ppm are injected on the next day of the preparation of samples and the % Amount found was calculated and %RSD was found to be 0.7 and chromatogram was shown in figure 3 (Table 3, Figure 8).

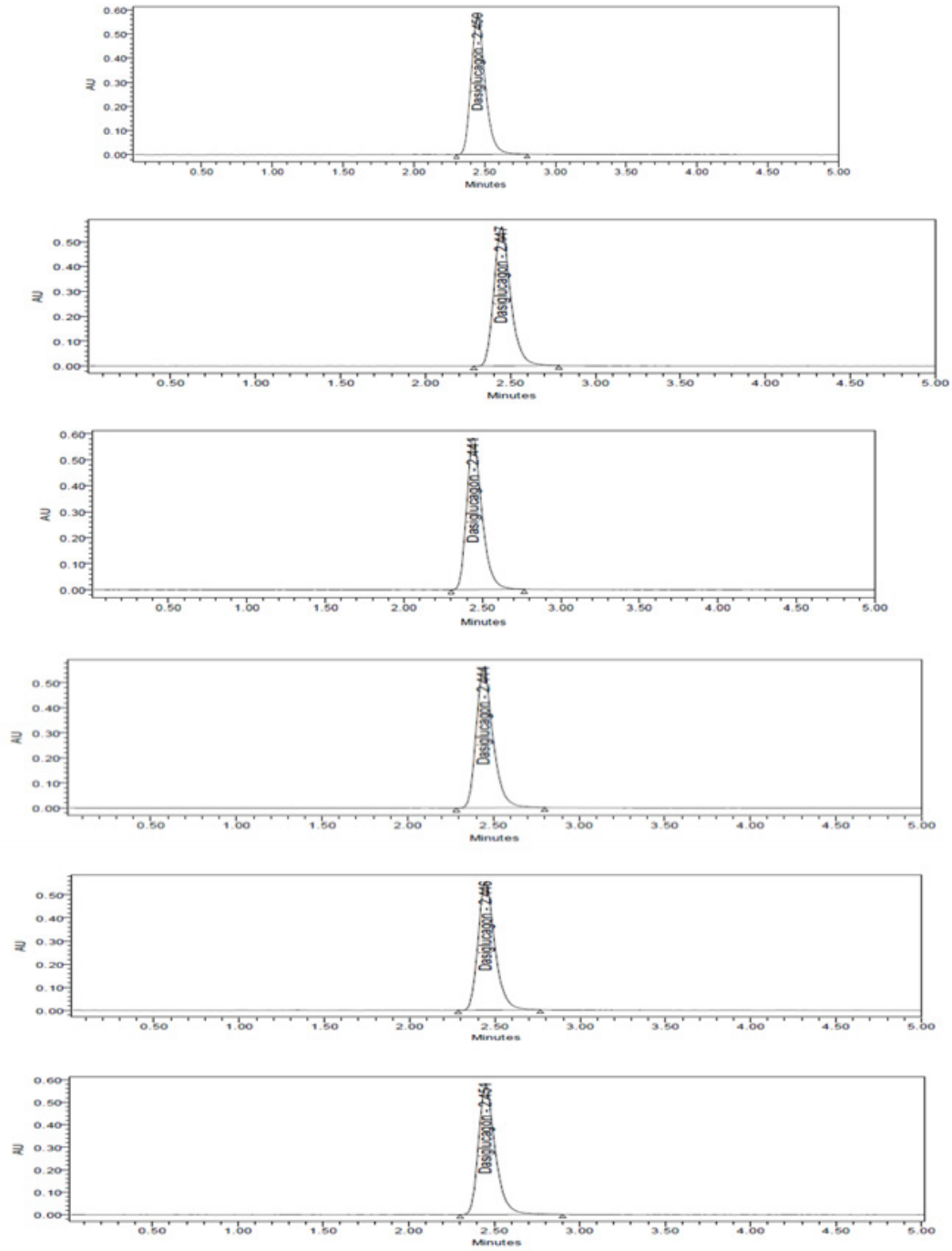

Figure 8: Repeatability Chromatogram.

Table 3: Intermediate precision data.

S. No	Peak Area
1	3576203
2	3553231
3	3576860
4	3588984
5	3574385
6	3521287

AVG	3565158
STDEV	24407.6
%RSD	0.7

Linearity: To demonstrate the linearity of assay method, inject 6 standard solutions with concentrations of about 3 ppm to 18 ppm of Dasiglucagon. Plot a graph to concentration versus peak area. Slope obtained was 332169 Y-Intercept was 7534.4 and Correlation Co-efficient was found to be 0.999 and Linearity plot (Table 4, Figure 9-15).

Table 4: Linearity Concentration and Response.

Linearity Level (%)	Concentration (ppm)	Area
0	0	0
25	3	1066416
50	6	1925671
75	9	3028643
100	12	3970103
125	15	4975021
150	18	6013506

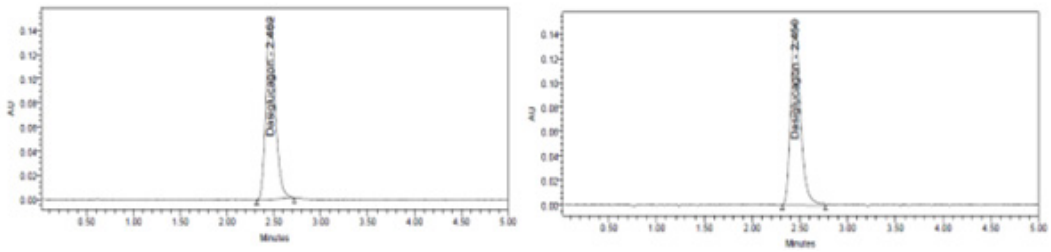

Figure 9: Linearity 25% Chromatogram.

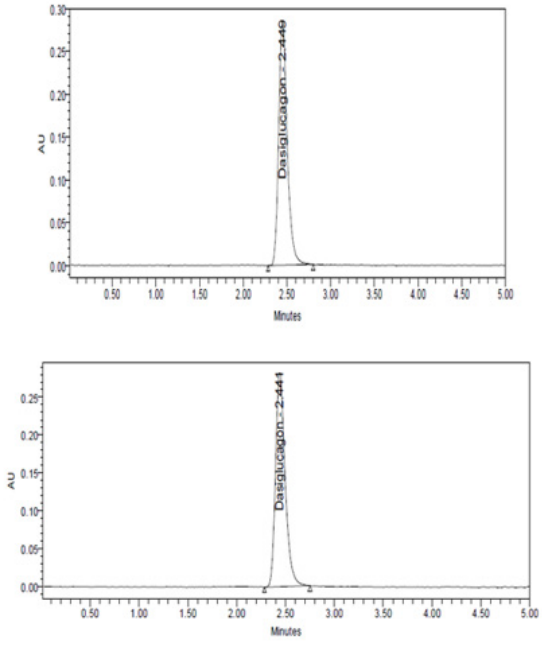

Figure 10: Linearity 50% Chromatogram.

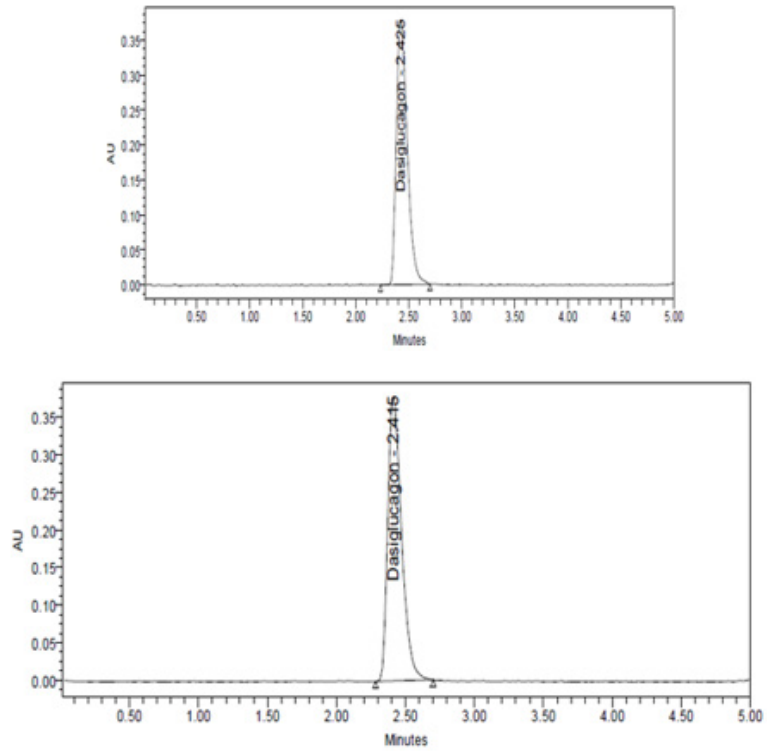

Figure 11: Linearity 75% Chromatogram.

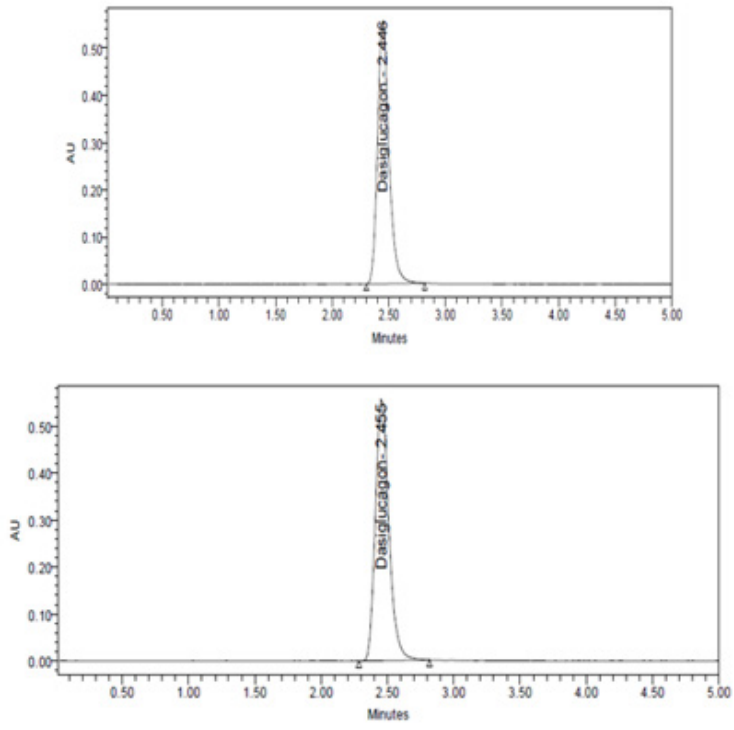

Figure 12: Linearity 100% Chromatogram.

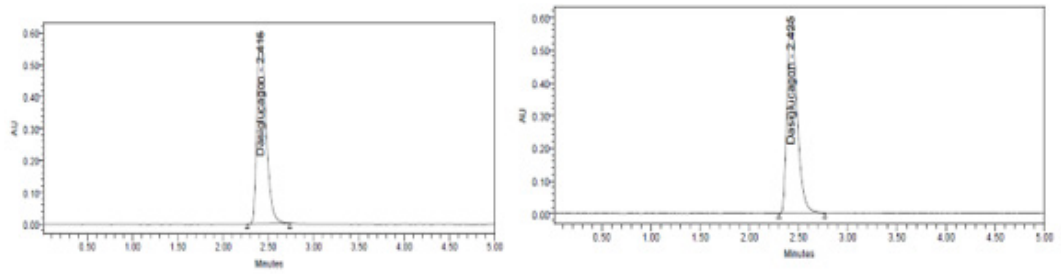

Figure 13: Linearity 125% Chromatogram.

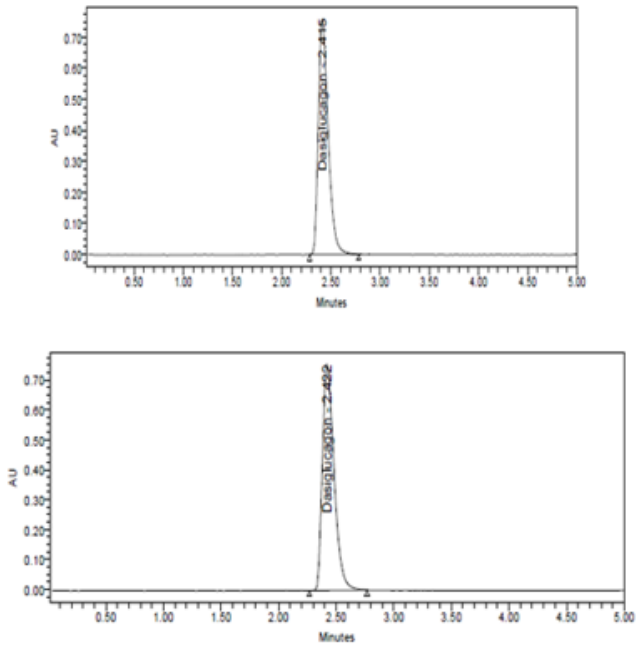

Figure 14: Linearity 150% Chromatogram.

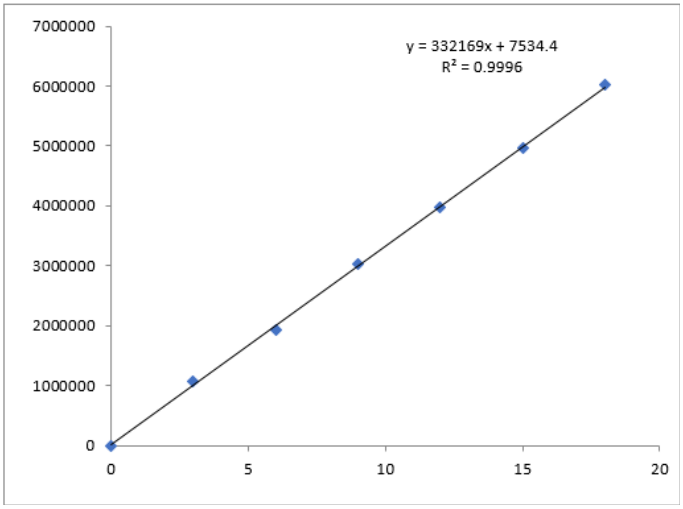

Figure 15: Linearity Plot.

Accuracy: Three Concentrations of 50%, 100%, 150% are Injected in a triplicate manner and %Recovery was calculated as 100.45. And chromatograms were shown in figure 6.11-6 (Table 5, Figures 16,17).

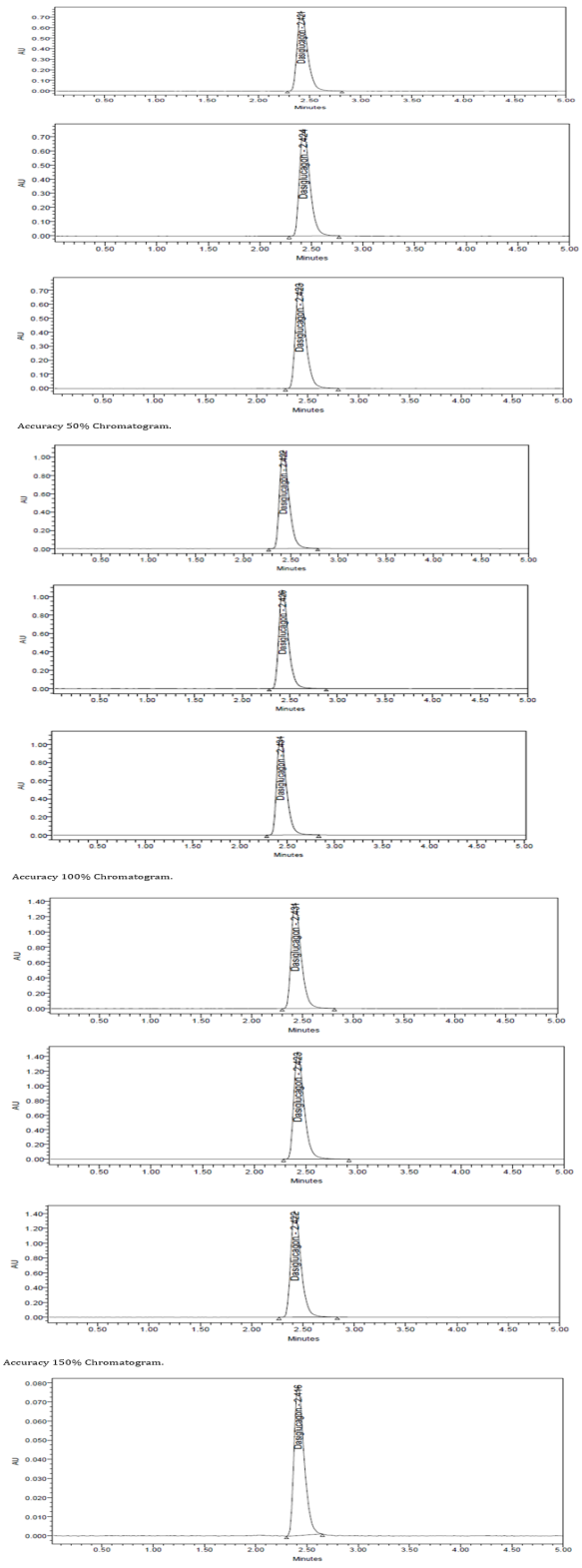

Figure 16: Linearity Plot.

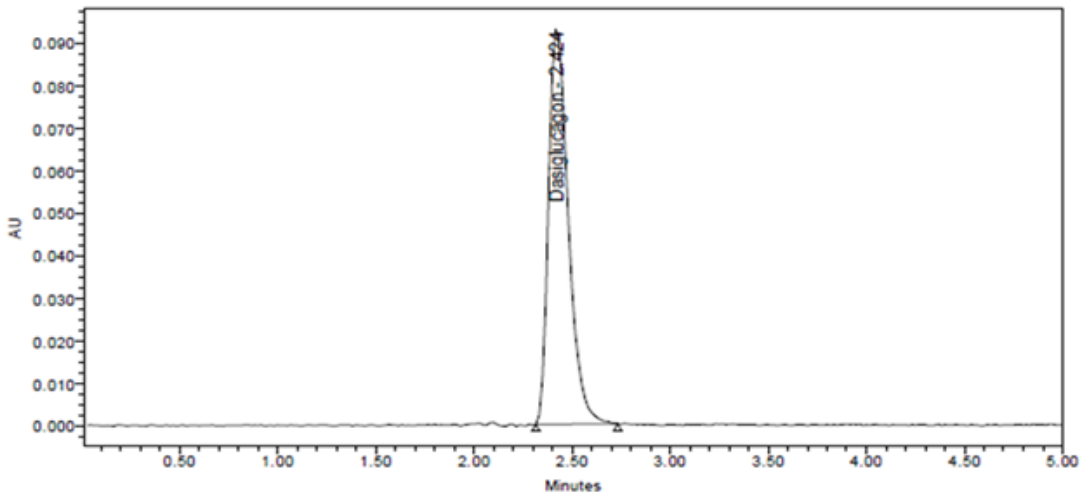

Figure 17: LOQ Chromatogram of Dasiglucagon.

Table 5: Accuracy data.

% Level	Amount Spiked (mg/mL)	Amount recovered (mg/mL)	% Recovery	Mean % Recovery
50%	50	50.54	101.09	100.45%
	50	50.53	101.06	
	50	50.02	100.03	
100%	100	101.87	101.87	
	100	101.74	101.74	
	100	100.73	100.73	
150%	150	148.1	98.73	
	150	149.86	99.91	
	150	148.41	98.94	

Robustness

Small Deliberate change in the method is made like Flow minus, flow plus, Mobile phase minus, Mobile phase plus, Temperature minus, Temperature Plus. %RSD of the above conditions are calculated (Table 6, Figures 18-23).

Table 6: Robustness Data.

Parameter	%RSD
Flow	-0.5
	0.5
Mobile phase	-1.1
	1.1
Temperature	-1.2
	1.2

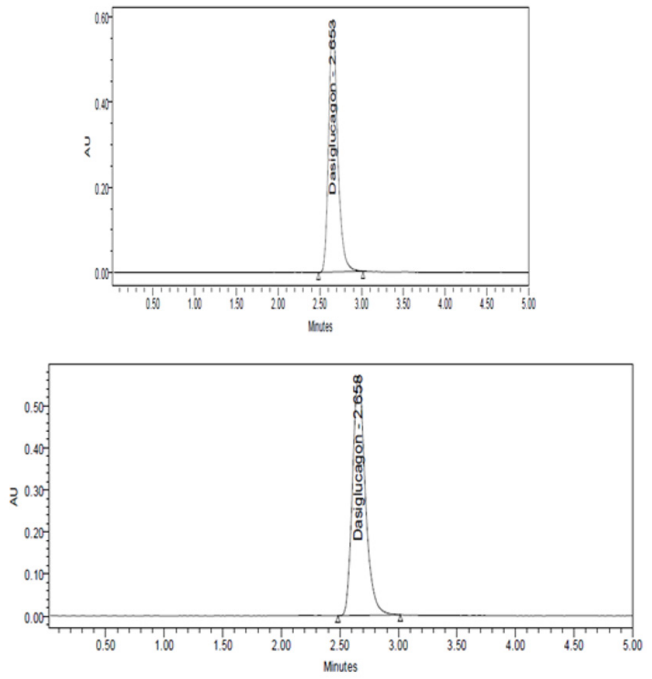

Figure 18: Flow minus Chromatogram of Dasiglucagon.

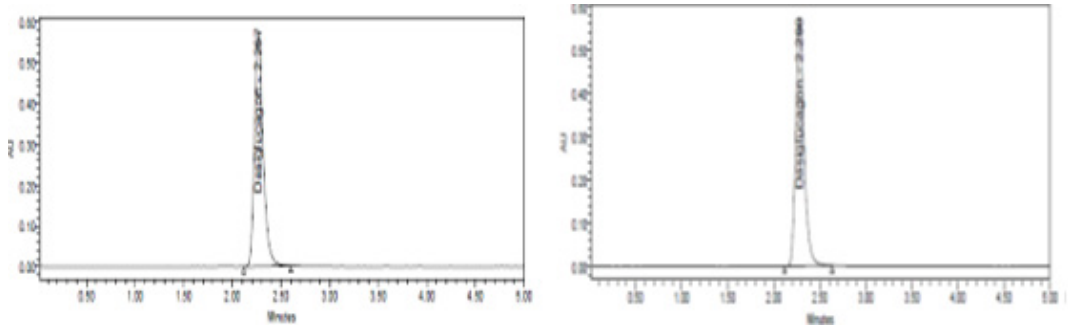

Figure 19: Flow plus Chromatogram of Dasiglucagon.

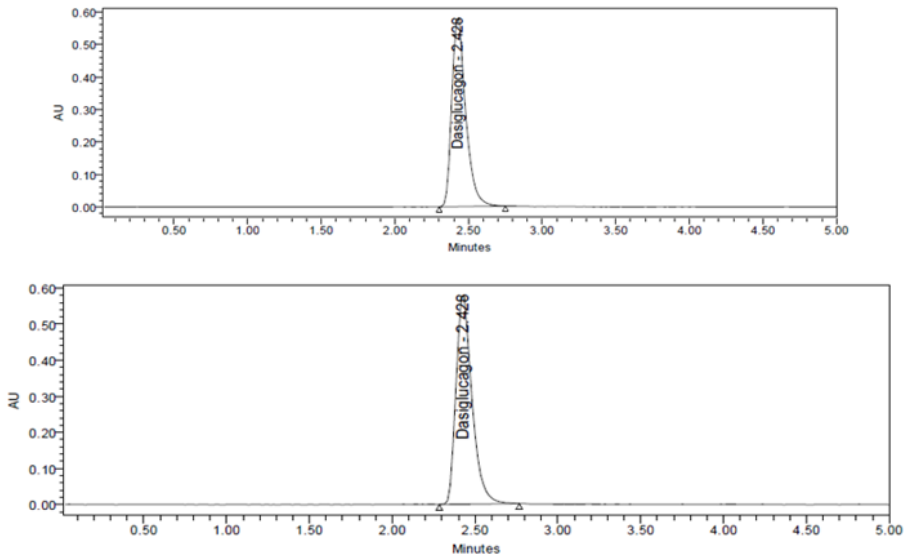

Figure 20: Mobile phase minus Chromatogram of Dasiglucagon.

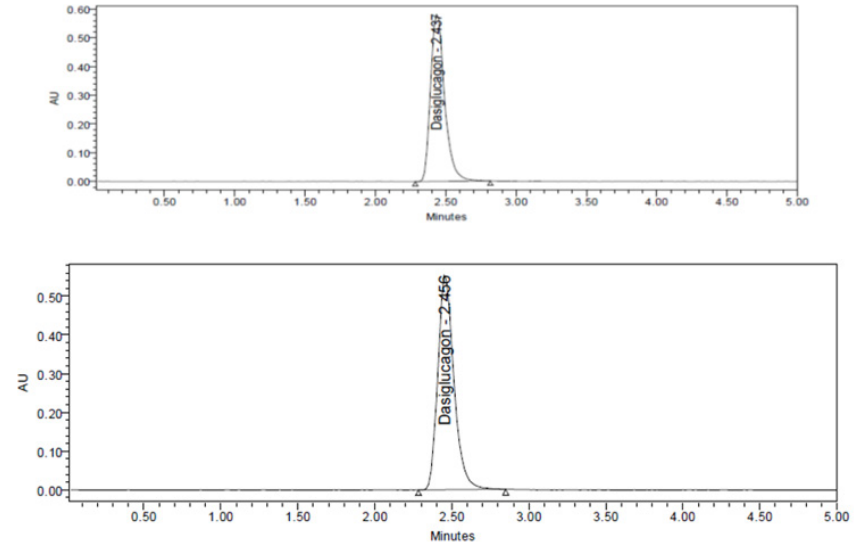

Figure 21: Mobile phase plus Chromatogram of Dasiglucagon.

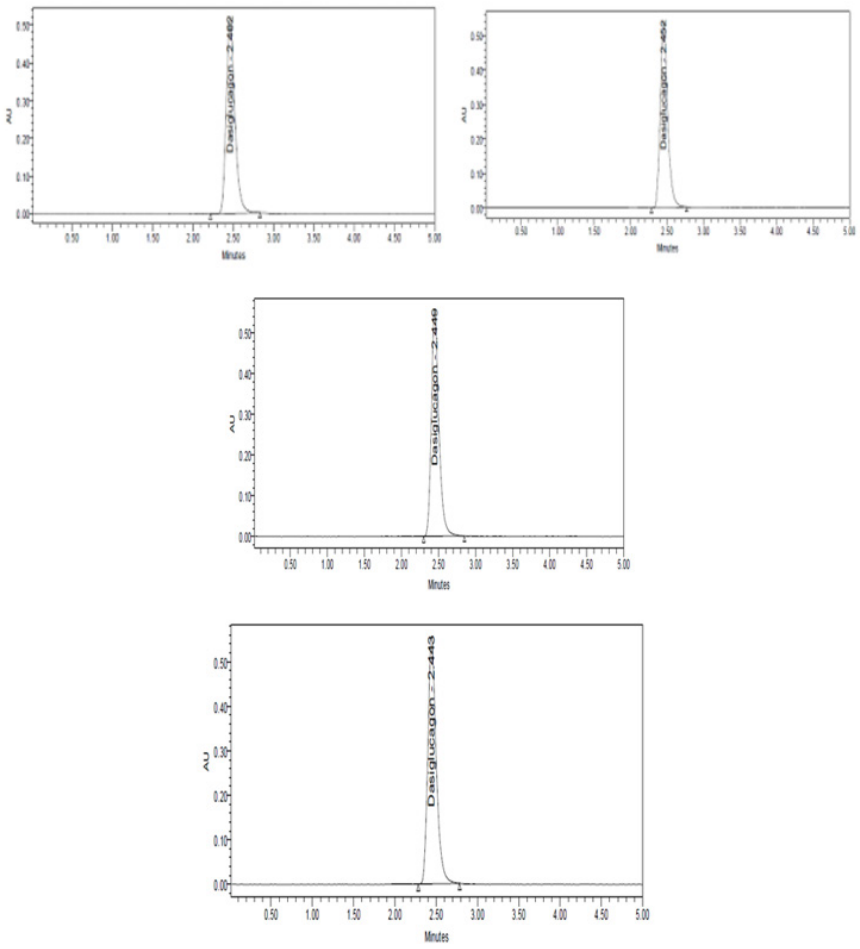

Figure 22: Temperature minus Chromatogram of Dasiglucagon.

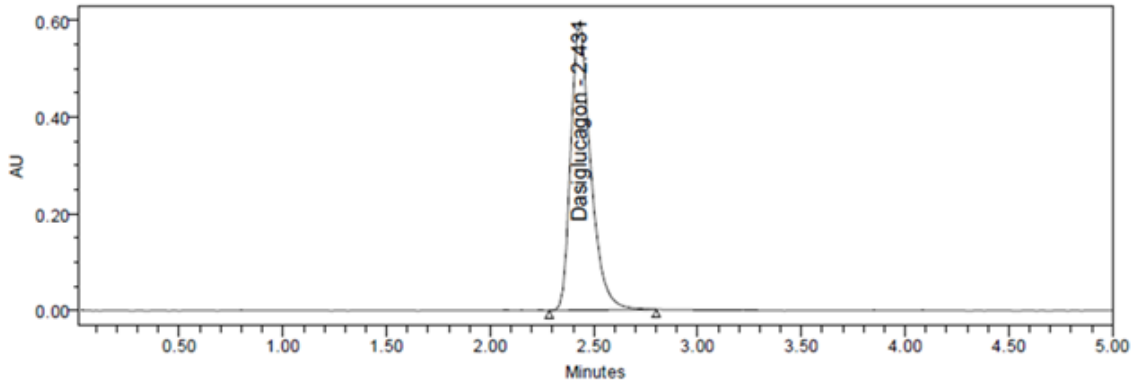

Figure 23: Temperature plus Chromatogram of Dasiglucagon.

Assay of Marketed Formulation

Standard solution and sample solution were injected separately into the system and chromatograms were recorded and drug present in sample was calculated using before mentioned formula (Table 7, Figure 24).

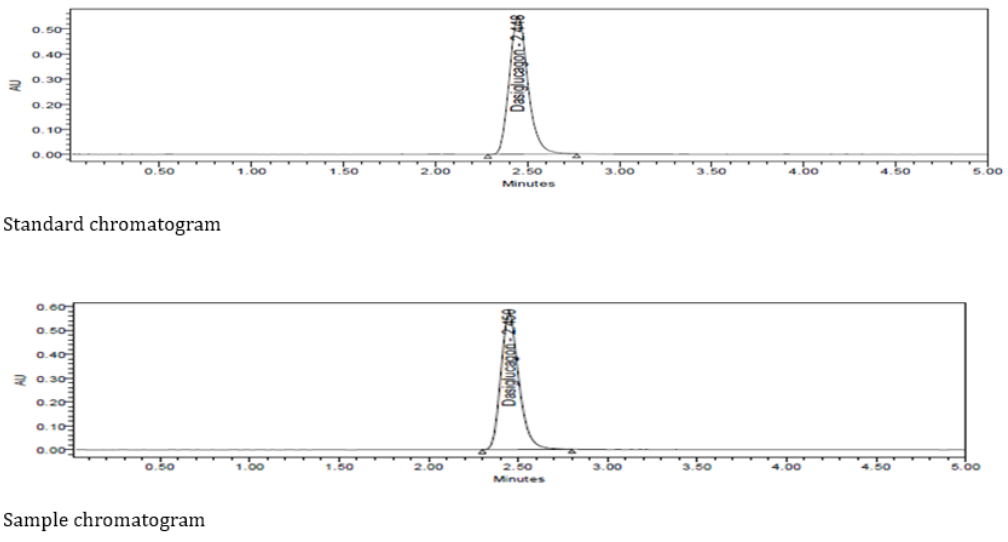

Figure 24: Assay Chromatogram.

Table 7: Assay of Formulation.

Sample No	%Assay
1	100.72
2	99.66
3	99.41
4	99.32
5	101.6
6	99.91
AVG	100.1
STDEV	0.89
%RSD	0.9

Summary And Conclusion

(Table 8) Chromatographic conditions used are stationary phase Ascentis C18 (150mm*4.6mm5m), Mobile phase Water: Methanol in the ratio of 60:40 and flow rate were maintained at 1.0ml/min, detection wave length was 230nm, column temperature was set to 30oC and diluent was mobile phase Conditions were finalized as optimized method. System suitability parameters were studied by injecting the standard six times and results were well under the acceptance criteria. Linearity study was carried out between 25% to150 % levels, R2 value was found to be as 0. 999.Precision was found to be 0.9 for repeatability and 0.8 for intermediate precision. LOD and LOQ are 0.027mg/ml and 0.082mg/ml respectively. By using above method assay of marketed formulation was carried out 100.11% was present. The retention time was found to be 2.16. Hence this method can be used for routine quality control analysis for this drug.

Table 8: Summary Table.

Parameters		Dasiglucagon	LIMIT
Linearity: Range (mg/ml)		3-18 mg/ml	R<1
Regression coefficient		0.999	
Slope(m)		39860	
Intercept(c)		7534.4	
Regression equation (Y=mx+c)		y = 39860x+7534.4.	
Assay (% mean assay)		100.18%	90-110%
Specificity		Specific	No interference of any peak
System precision %RSD		0.9	NMT 2.0%
Method precision %RSD		0.8	NMT 2.0%
Accuracy %recovery		100.10%	98-102%
LOD		0.027	NMT 3
LOQ		0.082	NMT 10
Robustness	FM	0.6	%RSD NMT 2.0
	FP	0.5	
	MM	1.1	
	MP	1.1	
	TM	1.2	
	TP	0.5	

Acknowledgement

None.

Conflict of Interest

None.

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