**Research Article** **CODEN: AJPAD7** **ISSN: 2321 - 0923**

**Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry**

**Journal home page: www.ajpamc.com**

https://doi.org/10.36673/AJPAMC.2020.v08.i04.A17

# PREPARATION OF GLUCAGON IMPURITIES RELATED TO LACTOSE FORMULATIONS

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## ABSTRACT

Glucagon is a peptide hormone consisting of 29 amino acids and is useful for maintenance of normal glycemia in blood[1]. Generally, peptide/proteins are highly sensitive to digestive enzymes present in the body, hence their administration is usually restricted to injection or nasal administration. To achieve enough stability of Glucagon, a suitable excipient needs to be chosen that could stabilize the peptide during freeze drying process and long-term storage as a dried formulation. Usually, Lactose is used as a stabilizer for freeze-dried formulations of glucagon and upon storage Glucagon will react with lactose via Maillard reaction and lead to formation of impurities. In our study, we prepared Glucagon lactose impurity, Glucagon di lactose impurity, and further purified them by using Preparative HPLC.

## KEYWORDS

Glucagon formulation, Lactose, Impurity profile, Stabilizer, Lactose impurity, Dilactose impurity and Preparative HPLC.

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## INTRODUCTION

Peptides have become increasingly important as therapeutic products ever since the advent of insulin therapy in 1920s. Currently, there are more than 60 approved peptide drugs and 150 peptide candidates in active clinical development and an additional 260 have been tested in human clinical trials[2].

Just as protein drugs, peptide drugs as well are susceptible to physical and chemical degradation and one of the usual approaches administered in achieving sufficient stability is to develop a stable

freeze dried formulation of the peptide with suitable choice of excipients to stabilize peptides during the freeze drying process and long term storage in dried formulations[3].

Typically, an essential stabilizer in the freeze-dried formulation of a protein is a non-reducing disaccharide such as sucrose or trehalose. These sugars can inhibit protein unfolding during the freezing and drying steps of freeze-drying, as well as provide a glassy matrix that is important for long-term storage stability of the dried product. For drugs that are formulated at acid pH, sucrose has the disadvantage of being susceptible to acid-catalyzed hydrolysis[4] forming reducing sugars glucose and fructose, which can chemically degrade proteins or peptides via the Maillard reaction.

Glucagon, a hormone secreted by the pancreas is a polypeptide consisting of a single chain of 29 amino acids and has a molecular weight of about 3485 Da. Both synthetic and recombinant glucagon have enough purity to be available for pharmaceutical use and as glucagon cannot be absorbed orally, it needs to be administered by injection.

Glucagon has an isoelectric point of 7.1 and is therefore insoluble in water at physiological pH (pH 4-8) and precipitates in pH-neutral aqueous solutions. In aqueous solutions of pH 3 or less, it is initially soluble but aggregates to form a gel within an hour. The aggregated glucagon is not suitable to be injected as it will clog a hypodermic needle and if administered might block blood vessels as well. To slow the aggregation process, an acidic formulation (pH 2-4) is commonly used to maintain glucagon in a relatively aggregation free state for a short period of time.

In addition to its physical instability, glucagon undergoes various types of chemical degradation. In aqueous solutions, it degrades to form several (at least 16 have been reported) degradation products[5]. In an acidic solution (pH 2-4) required to dissolve glucagon and prevent its aggregation, about 5-70% of glucagon decomposes into various degradation products within 24 hours at 37ºC[6].

In order to overcome glucagon's chemical instability, the currently available glucagon drug products (e.g, Glucagon for Injection) are lyophilized and produced as 2-part kits: one containing a freeze-dried formulation of glucagon with its excipient lactose and the other containing a syringe with diluent. Maillard reaction has been reported when Glucagon was formulated with the reducing disaccharide Lactose. In an acidic pH, lactose undergoes hydrolysis forming glucose[7].

Our objective was the preparation of the impurities formed in a freeze-dried formulation of Glucagon with Lactose as the stabilizer, i.e, Glucagon-lactose impurity and Glucagondilactose impurity.

## MATERIAL AND METHODS

### Reagents and Chemicals

Experimental section, unless otherwise stated, all reagents and solvents used in this study were commercially available. Glucagon, Lactose anhydrous, Dimethyl Sulfoxide and Methanol were used for the preparation of Glucagon Lactose impurity. After preparation, the impurities were further purified by Preparative HPLC and isolated by freeze drying. Water, Acetonitrile and Trifluoro acetic acid were used in buffer systems.

### Experimental Section

### Preparation of Glucagon Lactose and Dilactose Impurity

Charge Glucagon (1g) in an RBF. Charge Lactose anhydrous (0.479g). Charge Dimethyl sulfoxide (200ml) to the flask. Heat the reaction mass to 80-82ºC (40 min). Stir the reaction mass at a constant temperature (80-82°C) for an hour. Distill off reaction mass under vacuum at 80ºC (120 min). Cool the reaction mass to 25ºC (15 min). Charge Methanol (60ml) to the reaction mass and stir at a constant temperature (25°C) for an hour. Filter the solid and wash with methanol (5ml). Suck dry well for 5 min. Dry the solid under vacuum at 40ºC (6 hours) to get solid containing Glucagon lactose impurity and Glucagon di lactose impurity.

### Isolation and Purification of Glucagon Lactose and Glucagon dilactose impurity by Preparative HPLC

In a flask, charge solid containing Glucagon lactose impurity and Glucagon di lactose impurity (1g). Charge Mobile Phase 'A' (0.1% TFA in Water) solution of approx. pH 2.75 (200ml). Stir for clear

solution and filter through Hiflo. Wash the bed with Mobile Phase 'A' (50ml) solution. Load the materials onto column and run the column with Mobile phase A & B with the following Gradient composition.

Mobile Phase 'A' - 0.1% TFA in Water, Mobile Phase 'B' - Acetonitrile.

The system gradient method was set as follows (Table No.1) for the flow rate of 63 ml/min:

Collected fractions analyzed by HPLC and distilled off acetonitrile from fractions containing Glucagon lactose impurity and Glucagon di lactose impurity separately under vacuum at 40°C. The fractions were freeze dried under vacuum (70 hours) to get Glucagon lactose impurity around 90mg and Glucagon di lactose impurity around 70mg).

## RESULTS AND DISCUSSION

### Preparation of Glucagon Lactose and Dilactose impurity

There are 2 free amine groups present in glucagon. For preparation of Glucagon Lactose impurity, Lactose molecule gets added to the free amine 1 group in the above structure and for dilactose impurity, lactose molecules get added in free amine 1 and 2 positions in the glucagon structure.

Dry weight of compound obtained was about 1.17g. Required Glucagon Lactose impurity whose purity by HPLC was around 44% and for Dilactose impurity whose purity by HPLC was around 33%. The above impurities were further purified by preparative HPLC.

### Isolation and Purification of Glucagon Lactose and Dilactose impurity by Preparative HPLC

Glucagon Lactose, Glucagon di lactose impurity was purified by Preparative HPLC to get 86.73% for Glucagon Lactose and 69.82% purity for Glucagon di lactose impurity.

Chromatographic purity, Mass spectrum and NMR data (Bruker 600 MHz NMR Using Topspin software) is collected for impurities sample.

**Table No.1: Gradient method for Preparative HPLC**

| S.No | Time (min) | A (%) | B (%) |
|---|---|---|---|
| 1 | 0.00 - 8.00 | 80 | 20 |
| 2 | 8.01 - 28.0 | 74 | 26 |
| 3 | 28.01 - 48.0 | 73 | 27 |
| 4 | 48.01 - 62.0 | 73 | 27 |
| 5 | 62.01 - 70.0 | 71 | 29 |
| 6 | 70.01 - 80.0 | 70 | 30 |
| 7 | 80.01 - 90.0 | 10 | 90 |
| 8 | 90.01 - 100.0 | 80 | 20 |

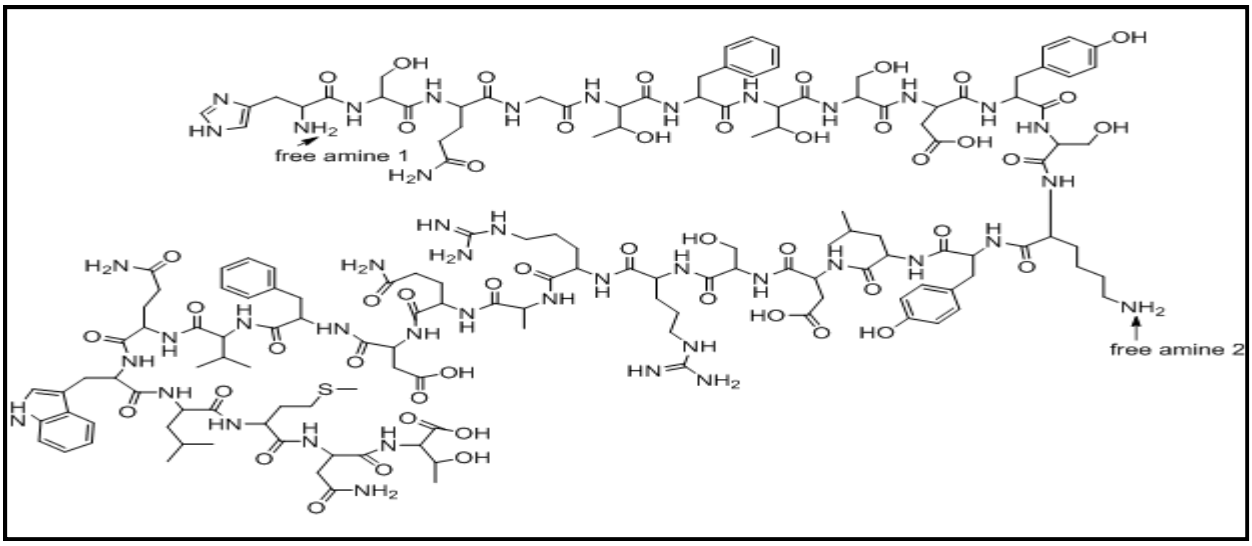


**Figure No.1: Structure of Glucagon**

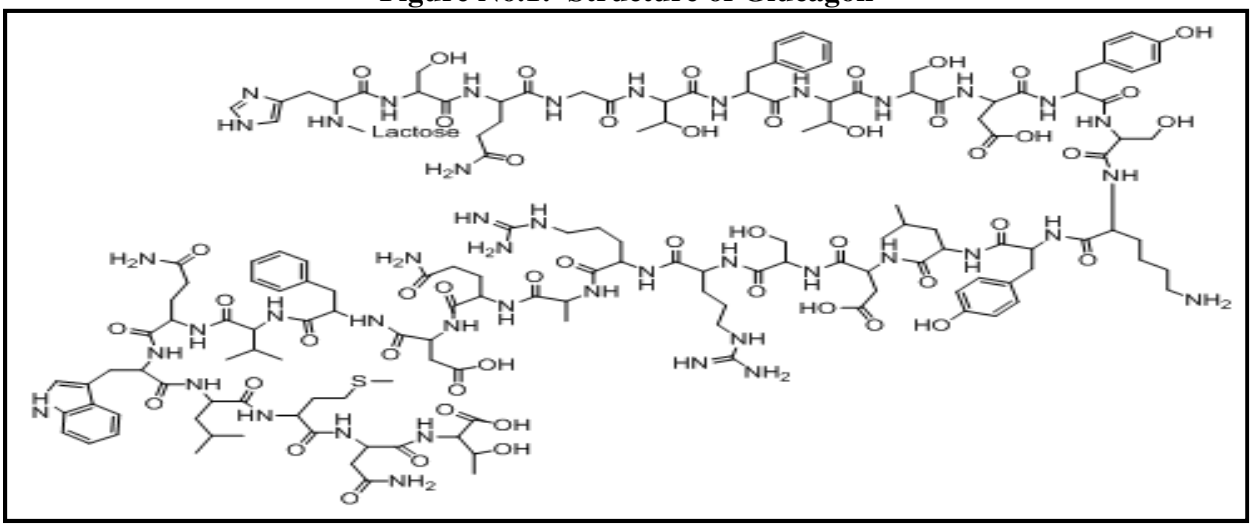


**Figure No.2: Glucagon Lactose Impurity**

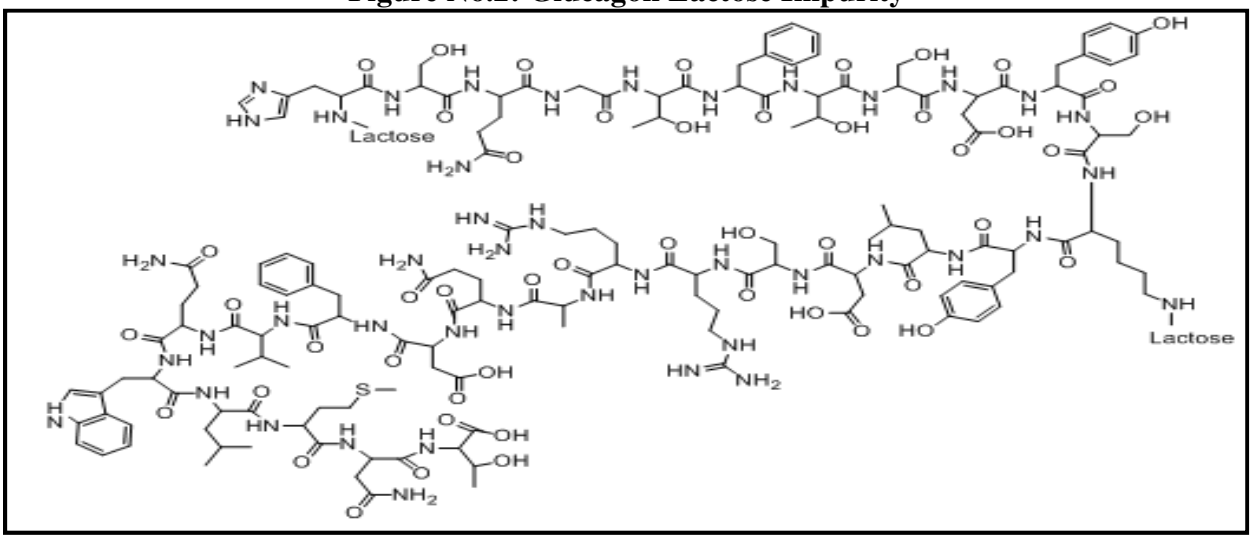


**Figure No.3: Glucagon Dilactose impurity**

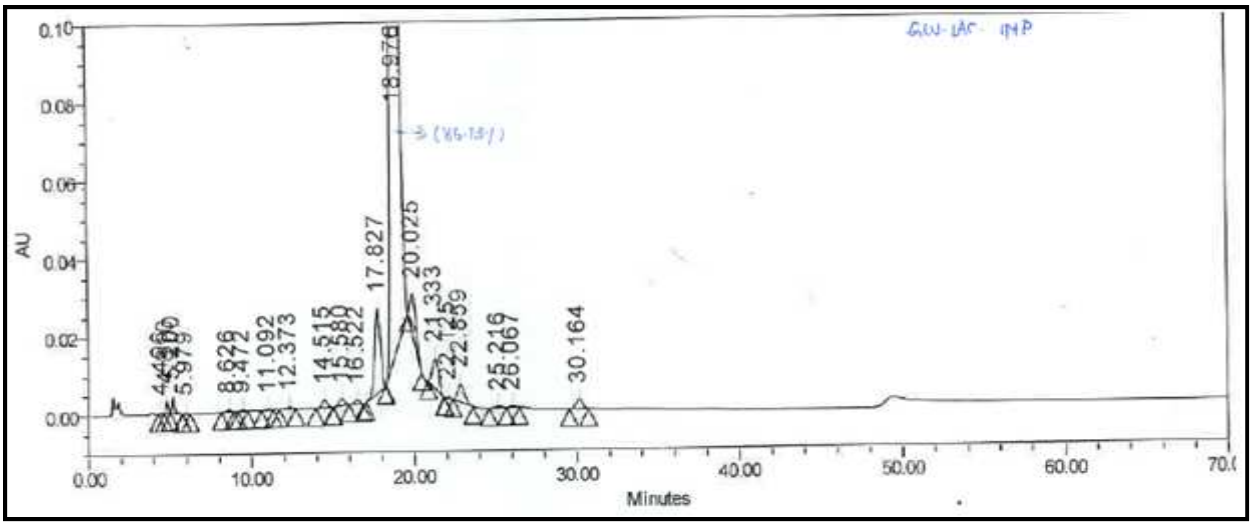


**Figure No.4: Chromatogram of Glucagon Lactose Impurity with purity of 86.73%**

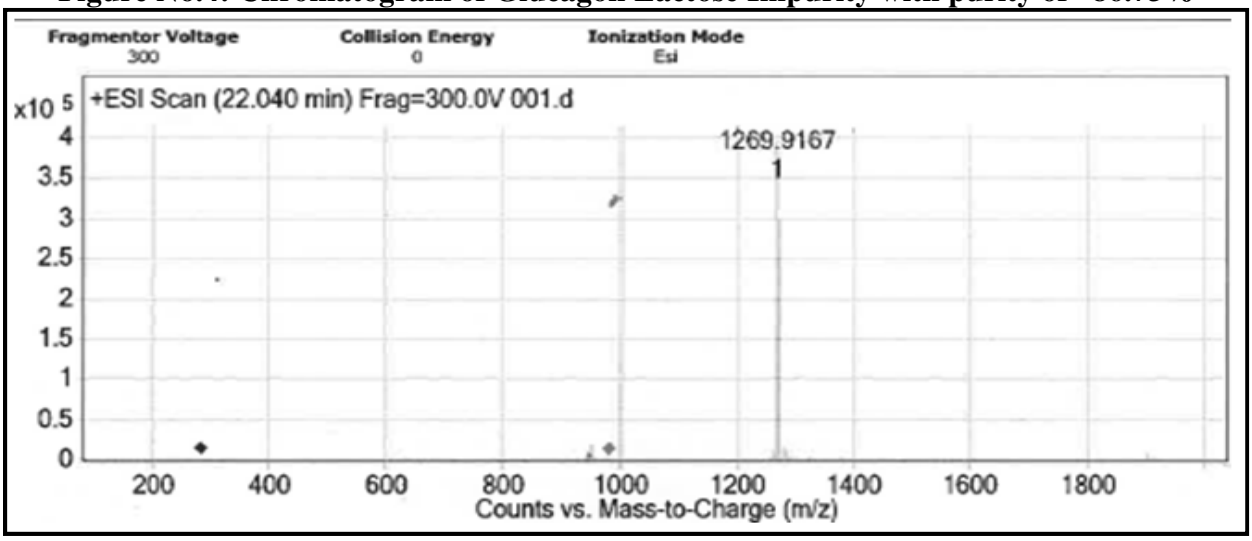


**Figure No.5: Mass chromatograph (M/3 + 1 data) of Glucagon lactose impurity**

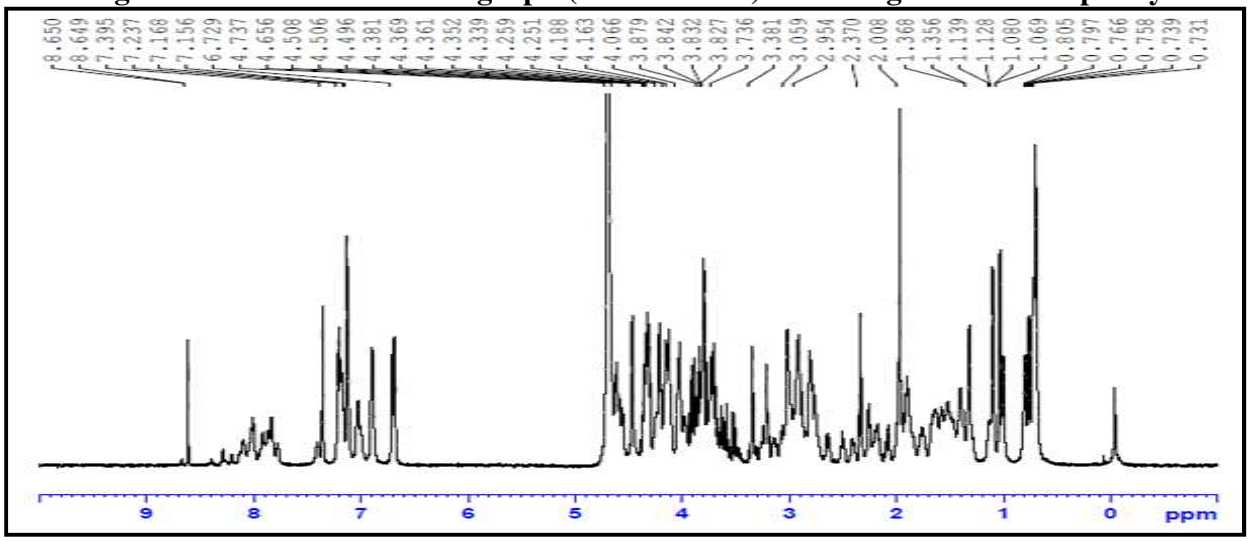


**Figure No.6: $^{1}$H NMR of Glucagon Lactose Impurity**

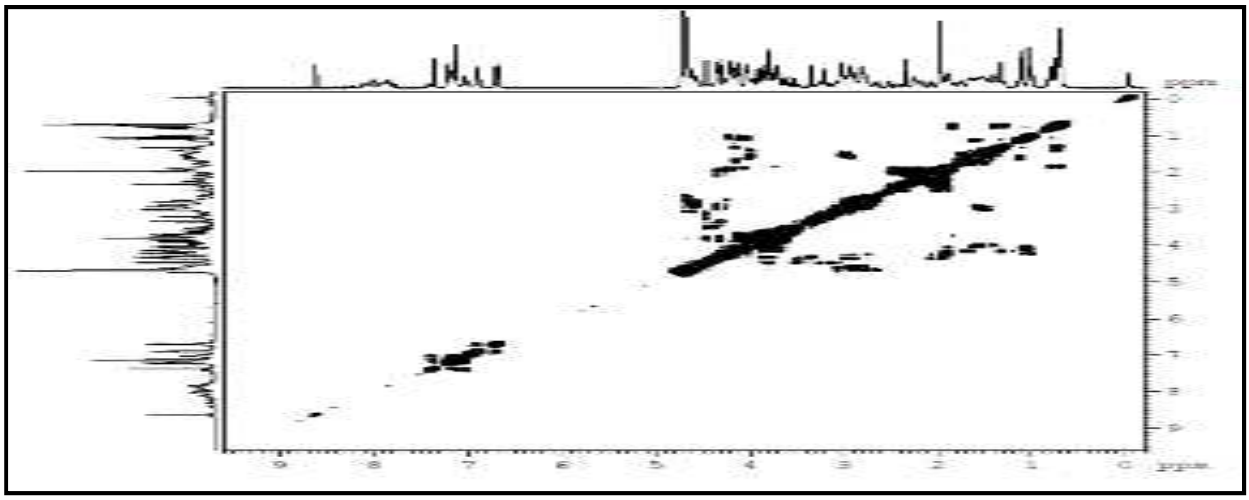

**Figure No.7: Homonuclear correlation spectroscopy (COSY) of Glucagon Lactose Impurity**

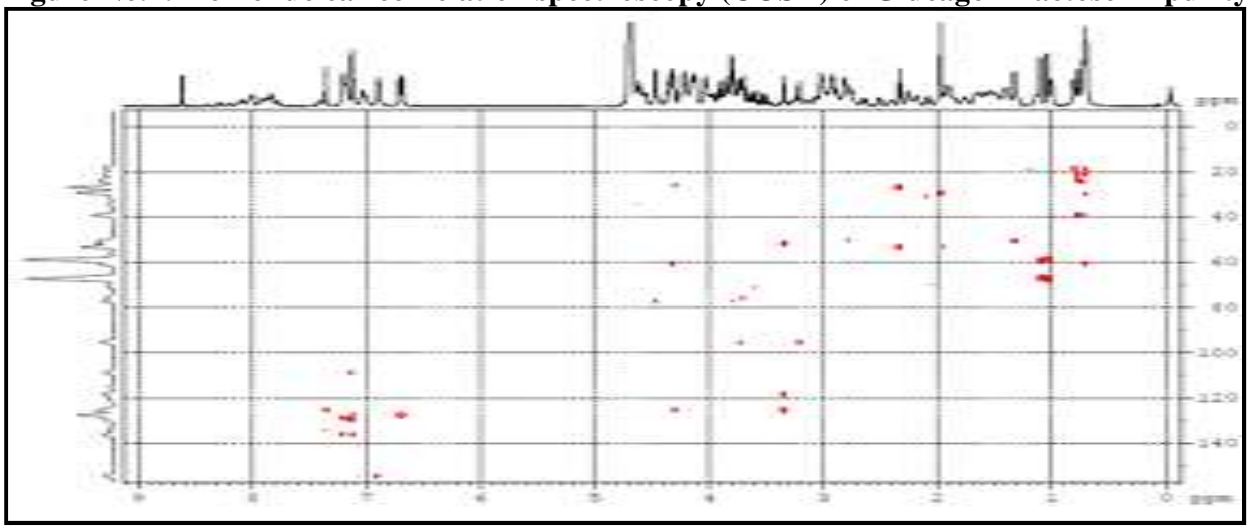

**Figure No.8: Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC) of Glucagon Lactose Impurity**

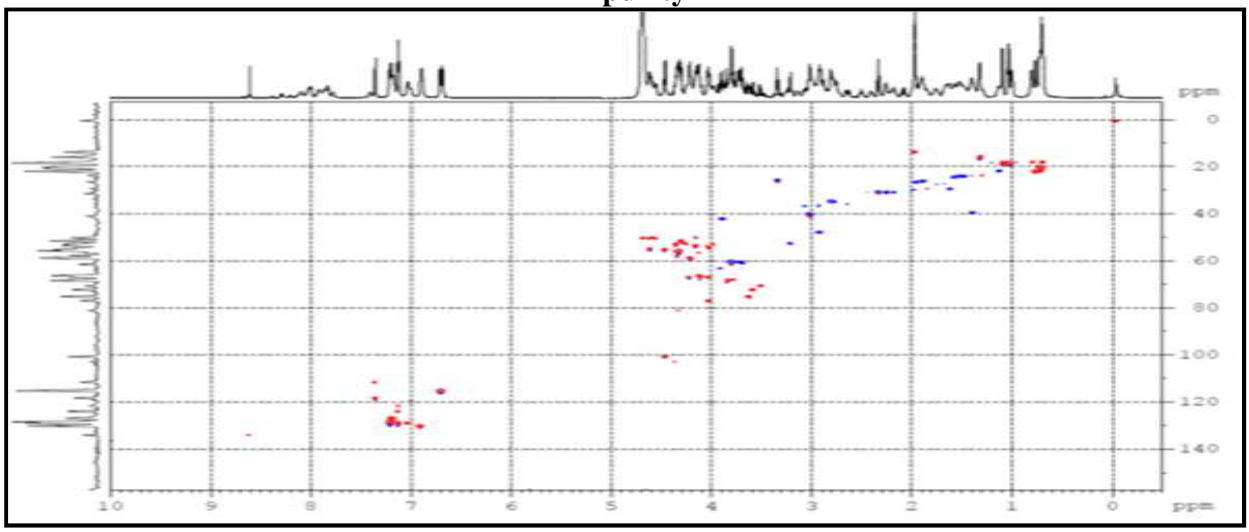

**Figure No.9: Heteronuclear single quantum coherence (HSQC) of Glucagon Lactose Impurity**

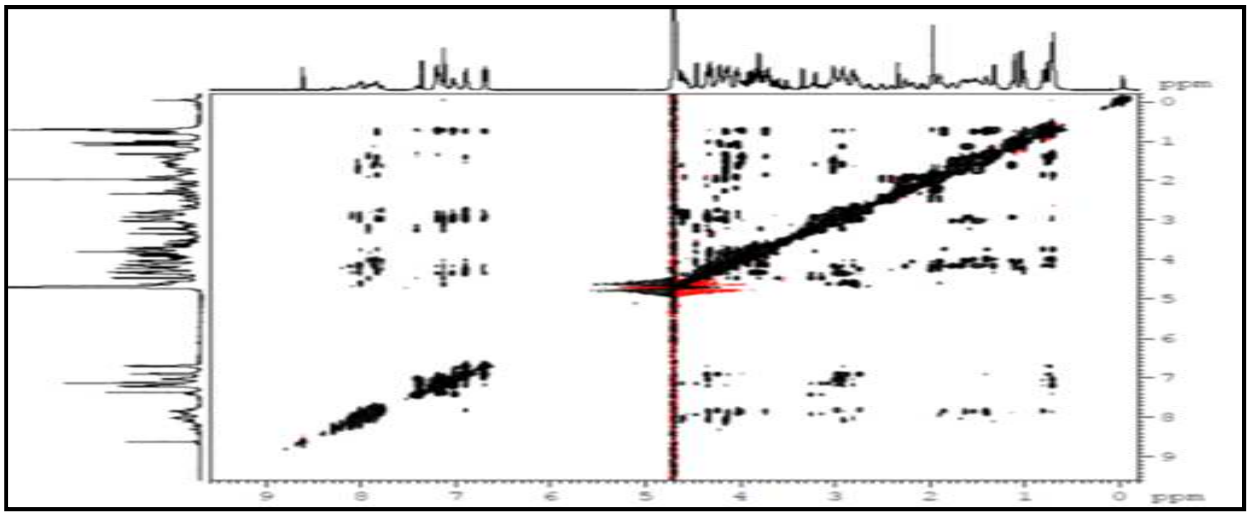


**Figure No.10: Nuclear over hauser Effect Spectroscopy (NOESY) of Glucagon Lactose Impurity**

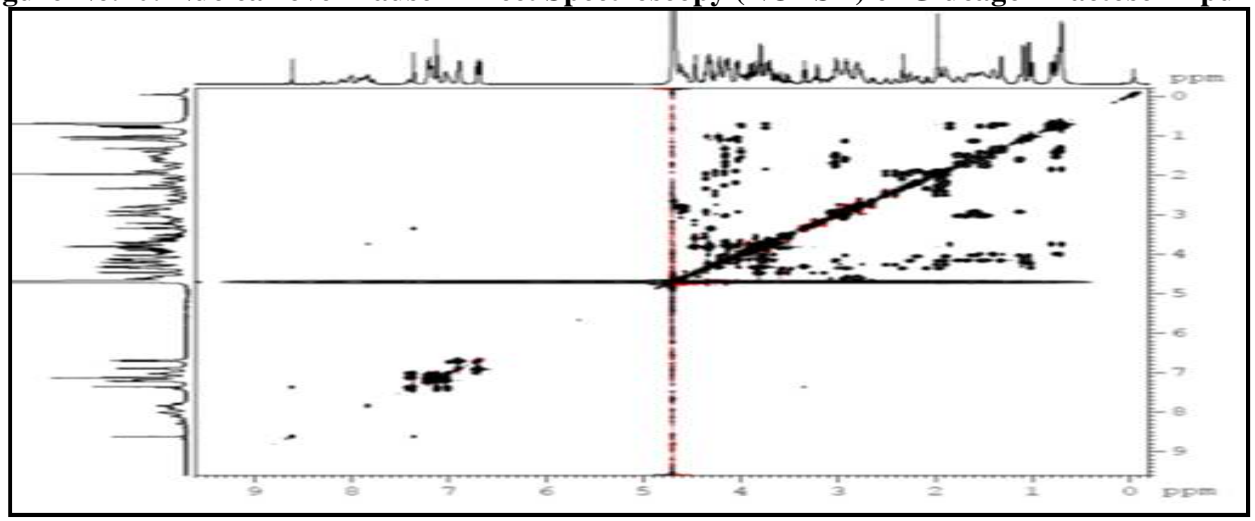


**Figure No.11: Total Correlation Spectroscopy (TOCSY) of Glucagon Lactose Impurity**

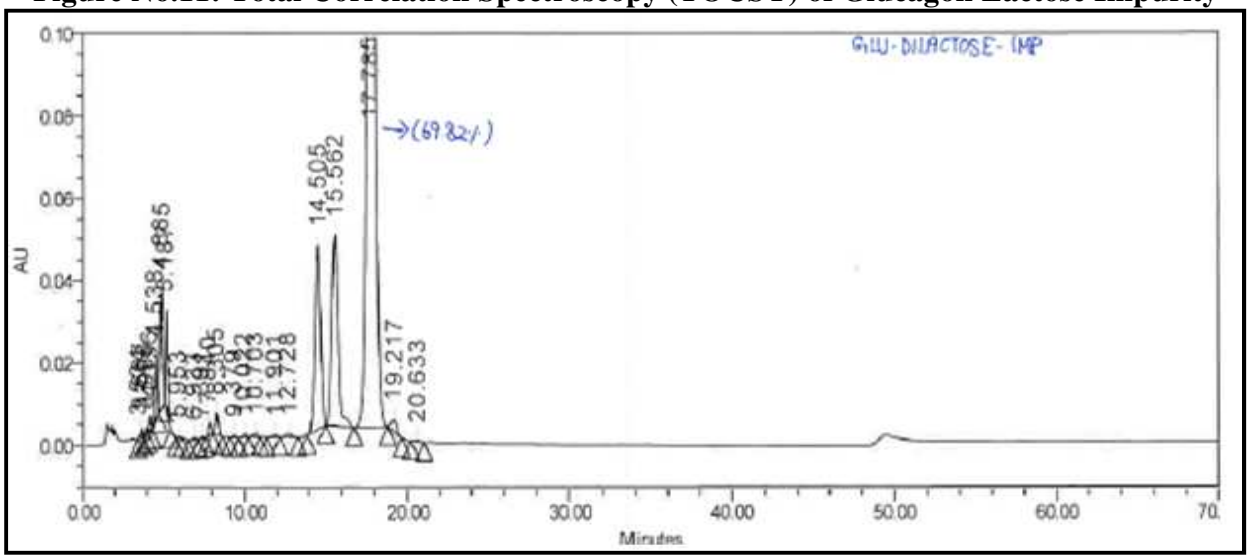


**Figure No.12: Chromatogram of Glucagon Dilactose Impurity with purity of 69.82%**

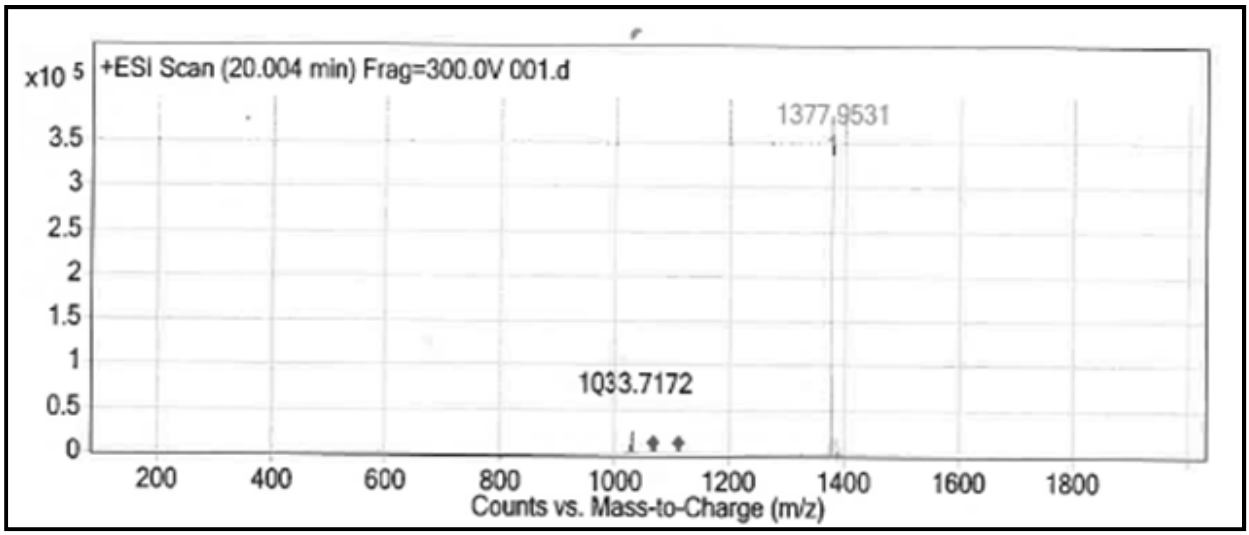


**Figure No.13: Mass chromatograph (M/3 + 1 data) of Glucagon di lactose impurity**

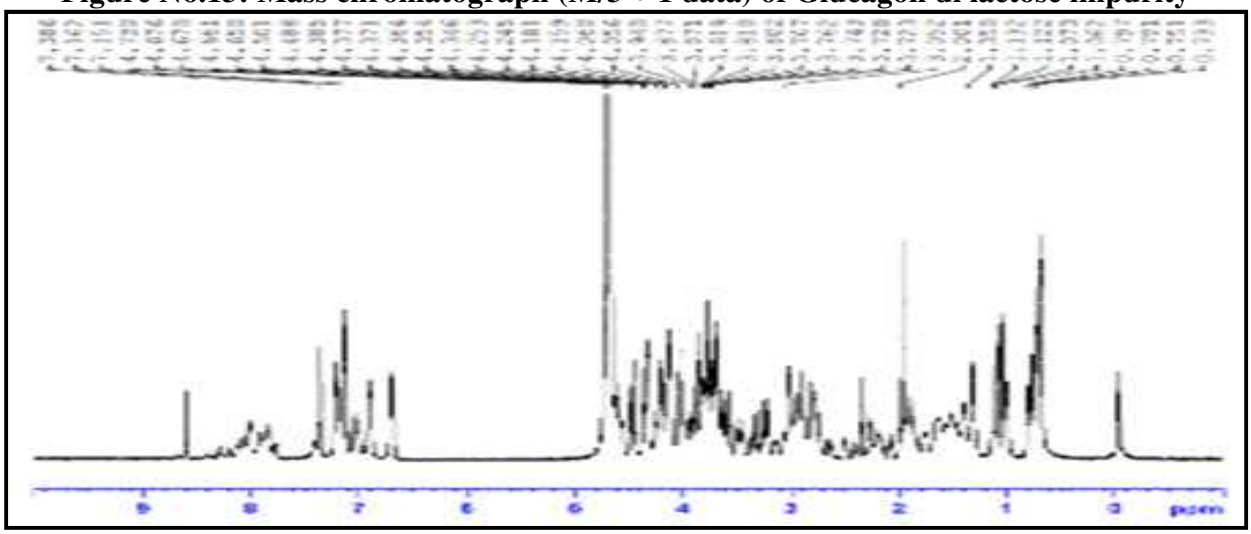

**Figure No.14: $^{1}H$ NMR of Glucagon di lactose impurity**

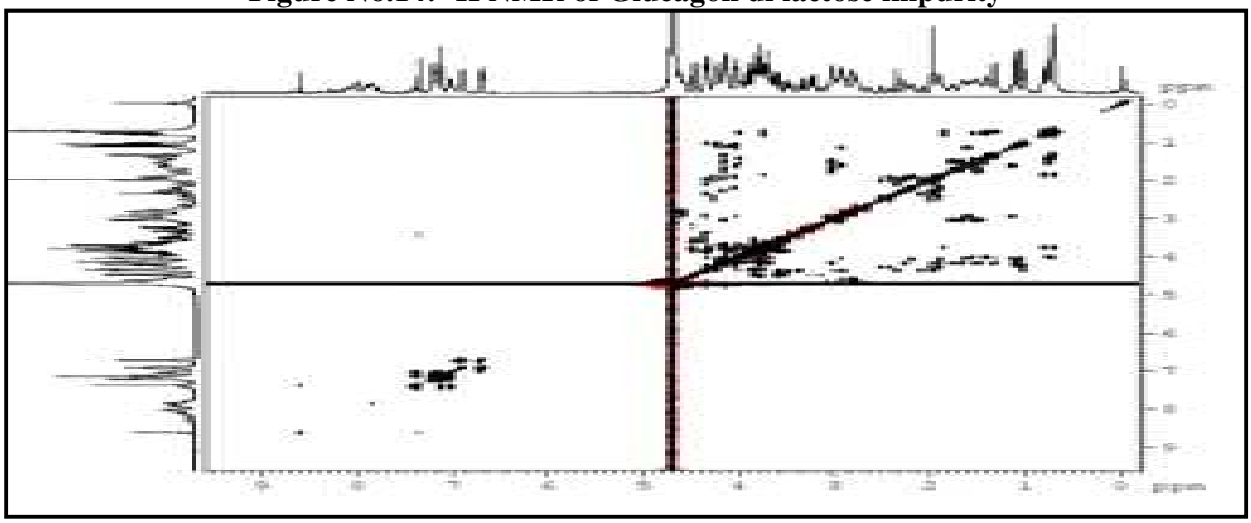

**Figure No.15: Homonuclear correlation spectroscopy (COSY) of Glucagon di lactose impurity**

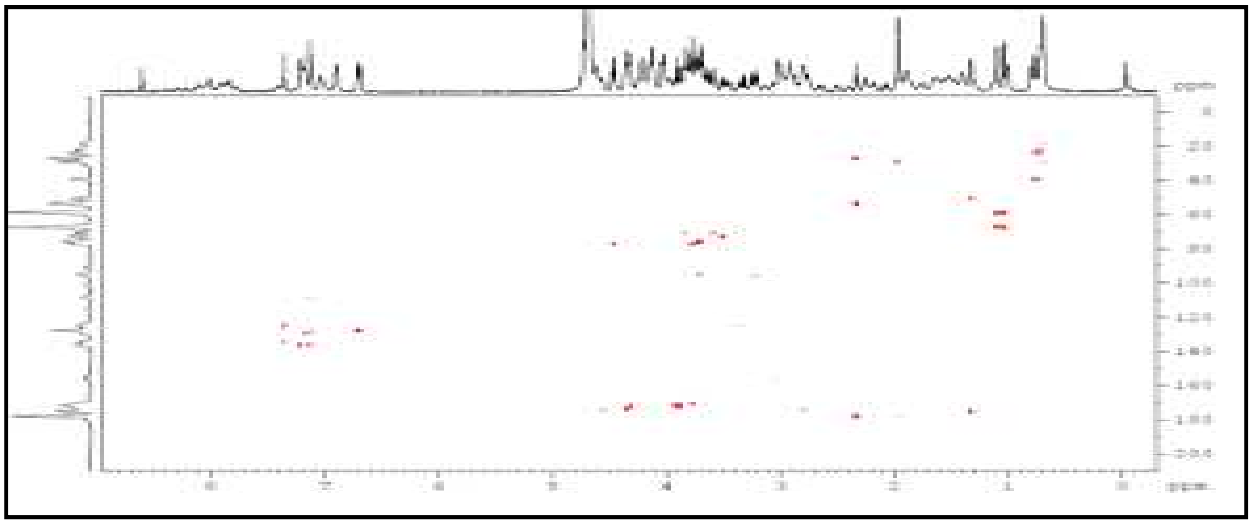

**Figure No.16: Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC) of Glucagon di lactose impurity**

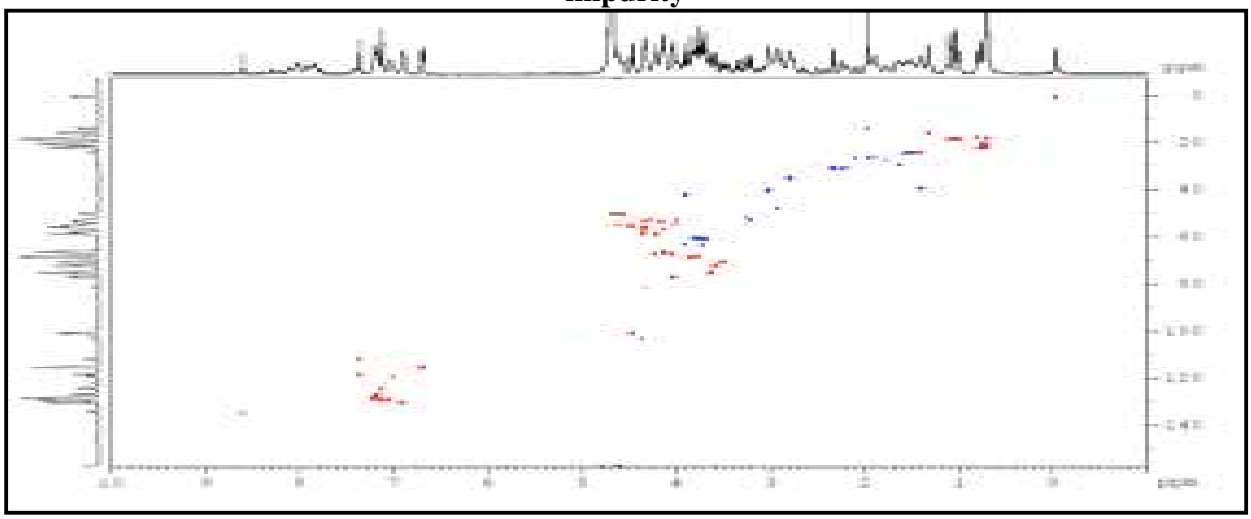

**Figure No.17: Heteronuclear single quantum coherence (HSQC) of Glucagon di lactose impurity**

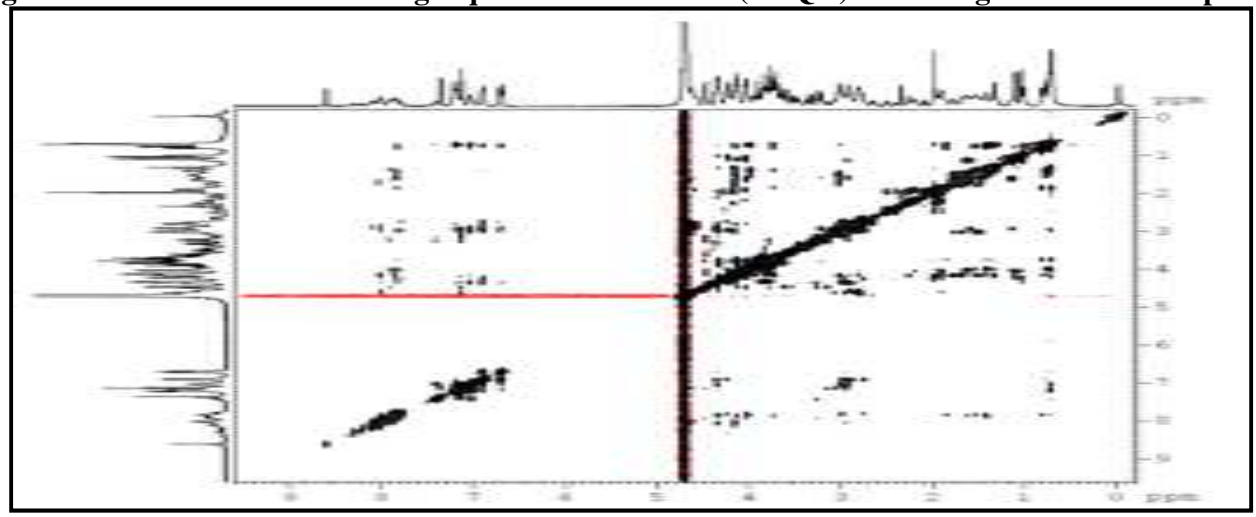

**Figure No.18: Nuclear Overhauser Effect Spectroscopy (NOESY) of Glucagon di lactose impurity**

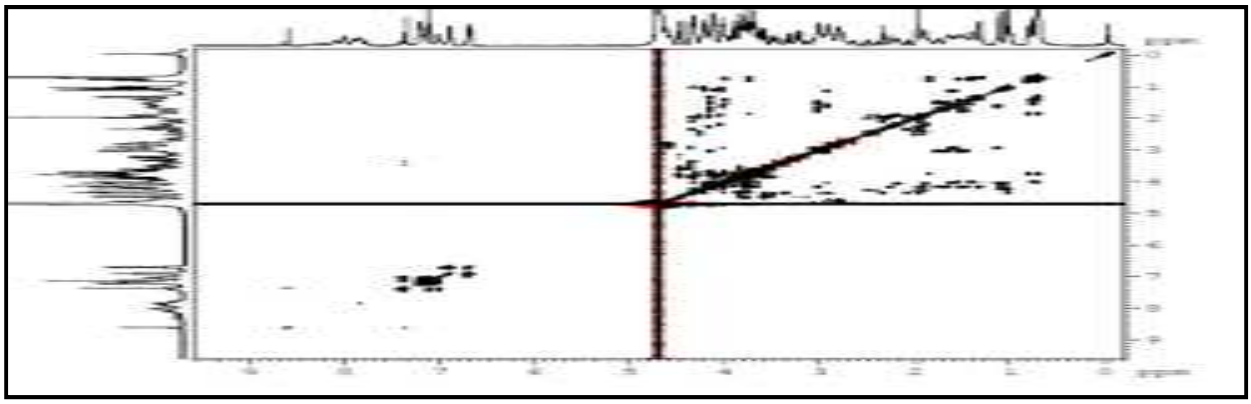

**Figure No.19: Total Correlation Spectroscopy (TOCSY) of Glucagon di lactose impurity**

## CONCLUSION

Glucagon Lactose Impurity and Glucagon Dilactose impurity were successfully prepared and purified using Preparative HPLC with final purity as follows: Glucagon Lactose Impurity around 87%, Glucagon Di lactose Impurity around 70% purity.

## ACKNOWLEDGEMENT

Our group is thankful to Department of Scientific and Industrial Research India, Sanjeev Sethi (COO and Chief Scientific Office Mylan Inc) ; Dr. Abhijit Deshmukh (Head of Global OSD Scientific Affairs); Jyothi Basu {Head - Global API (Active Pharmaceutical Ingredients)}; Ajay Singla Head of Global Injectables R and D and Scientific Affairs Bangalore-R and D; Dr Chandra Has Khanduri (Head of Global API R and D and Scientific Affairs and R and D) ; Dr Sureshbabu Jayachandra ( Head API R and D ); Dr Arvind (Head of Analytical Department MLL API R and D) as well as analytical development team of Mylan Laboratories Limited for their encouragement and support. We would also like to thank Dr. Narahari Ambati (AGC- India IP) and his Intellectual property team for their support.

## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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**Please cite this article in press as:** Madhuresh Kumar Sethi *et al.* Preparation of glucagon impurities related to lactose formulations, *Asian Journal of Pharmaceutical Analysis and Medicinal Chemistry,* 8(4), 2020, 137-146.