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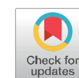
Simultaneous characterization of insulin HMWP and protamine sulphate in complex formulations through SEC-coupled mass spectrometry



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High molecular weight protein aggregates present in a recombinant human insulin and analogs are conventionally quantified by SEC-HPLC and identified by SEC-MALS as oligomeric population which lacks precise identification of species. The limitation of these two techniques is vanquished though simultaneous separation and identification by SEC coupled with MS. The identification was established with organic solvent based isocratic elution followed by MS for parallel separation and identification of HMWP species. The developed SEC-MS method is validated to establish the method capability and variability. Further investigations under stress conditions of Insulin analogues revealed the capability of the method to separate higher order oligomeric (Trimeric, and Tetrameric) species alongside covalent dimeric species. Additionally, the method holds good in separating and sequencing protamine peptides used in suspension (Neutral Protamine Hagedorn) and biphasic/mixed (70/30) formulations of Human insulin using ETD-MSMS. The data presented here shows insight towards utilization of state-of-the-art SEC-MS technique in the biopharmaceutical field as a tool to establish the structural comparability of higher order species in biosimilars and to investigate the lot to lot batch variability for protamine sulphate in-terms of sequence confirmation.

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1. Introduction

Human insulin, the first approved recombinant pharmaceutical is a polypeptide composed of 51 amino acids. Insulin consists of A-chain (21 amino acids) and B-chain (30 amino acids), that are linked by two disulfide bridges (A7-B7 and A20-B19). An additional disulfide bond A6-A11 is also present in A-chain. The monomeric structure of insulin is the active form for the treatment of diabetes mellitus that binds to the receptor and initiates the signaling cascade, leading to sugar absorption. Pharmaceutical formulations of human insulin are very critical for stabilizing the monomer (biologically active form) by shifting equilibrium towards higher order species (hexamer) that reduces the formation of covalent (or non-covalent) aggregates in drug product [1]. Numerous stresses (e.g., temperature fluctuations, light, shaking, surfaces, pH adjustments, etc.) can induce protein aggregation during different stages of production, storage or shipment [2].

One of the critical quality attributes of human insulin listed in United States and European Pharmacopoeia monograph is high molecular weight products (HMWPs), which are defined by pharmacopeia as covalent impurities larger than the insulin monomer in active pharmaceutical ingredient (API) and drug product.

Low content of HMWP in insulin pharmaceutics ensures less exposure to diabetic patients, however, it is inevitable to fully prevent as they get formed during use and storage of insulin products. Insulin HMWP is a blend of so far less characterized species but has been found to have reduced target binding and functional activity [3]. While precise function and dysfunction of insulin aggregates is not well understood, several studies have reported that the levels of insulin aggregate may increase in circulation over a period suggesting lower clearance of aggregates in comparison to monomeric insulin [4].

Covalent insulin aggregates (dimers) are reported to be primarily formed by covalent linkage between A21 Asn to either B1 Phe or NH_2 terminal Glycine by Hjorth, C. F et al. In the published study they characterized the HMWP species structurally, biophys-

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ically, and biologically and reported that HMWP has no or minimal biological activity and presence of HMWP is unlikely to have a negative impact on the physical stability of human insulin formulations [5]. A proposed mechanism for the formation of covalent insulin dimers was constructed based on a reactive anhydride intermediate formed at A21Asn [6,7]. The initial formation of the anhydride intermediate would result in a mass loss of 17.03 Da (NH_3). Upon formation of the reactive anhydride intermediate, two possible reactions can occur. One, addition of water would result in a deamidation reaction and second, nucleophilic attack on the anhydride intermediate by an amino group would form a covalent cross link between two insulin molecules leading to covalent dimer formation [7].

The conventional way for estimation of HMWP is through size exclusion chromatography as is described in pharmacopeia of various regulatory organizations. The method allows highly accurate quantitation; however, the salt buffers and mobile phases eludes direct characterization of these species through mass spectrometry. For the same reason, HMWP and/or aggregates in biologic drugs are often characterized by light scattering techniques such as multiangle light scattering (MALS) or dynamic light scattering (DLS) couple with Size exclusion chromatography [8]. While light scattering techniques provide an overall information, they are marred by limitation in providing accurate molecular weight as well as detailed information. Considering these challenges, developments towards more accurate and precise characterization of protein aggregates and/or antibody drug conjugates (in its denatured or native state) or vaccines have been carried out where result outcomes were obtained by coupling high resolution mass spectrometry to SEC or LC [9–16]. All these studies were published for large biopharmaceutics and to our knowledge no information is currently available for direct assessment of high molecular weight species in small peptide or protein-based therapeutics.

Recombinantly produced human insulin marketed by Eli Lily as Humulin® is commercially available in three different formulations: soluble (Regular or R), suspension (Neutral protamine Hagedorn or NPH or N) and biphasic (mixed or 70/30). All have similar biological function but different pharmacodynamics – with short to intermediate therapeutic regimen [17]. For insulin suspension (Neutral Protamine Hagedorn) and mixed formulations (70/30), protamine sulphate is used as one of the excipients for crystallization (which leads to prolong action) during preparation of the drug product. Because of its biological origin it is of utmost importance to have a detailed understanding of protamine sulphate. The active substance protamine sulphate is a purified mixture of highly basic (rich in Arginine) peptide derived from the sperm nuclei of chum salmon fish off the coast of Japan (a specific area off the coast of Honshu). Recently and due to the fishing restrictions in Japan following the earthquake and the tsunami in March 2011, sourcing of the raw material has been done in other fishing grounds (coast off Hokkaido). This new natural raw material has shown endogenous heterogeneity as shown by Gucinski et al. [18].

USP grade Protamine sulphate is known to be a mixture of four arginine-rich peptides with amino acid sequences of approximately 30 amino acids. The average molecular mass for each peptide is estimated to be 4–5 KDa [18]. Each of these peptides predominantly contain Arginine with a few Alanine, Valine, Serine, Proline and Glycine residues. Sequence variations may occur in amino acid sequences due to differences among individual fish from the separate geographical populations, and/or differences in methods of extraction and purification [18]. Thus, sequence characterization of protamine sulphate used for Insulin are of utmost importance for characterization of insulin suspensions and mixed formulations. Routine analysis of identifying quality or heterogeneity of protamine peptides is by conventional HPLC method, which lacks precise identification of species. Due to high salt content present in mobile phase, this method is not compatible for characterization through mass spectrometry.

This study aimed at direct identification and characterization of HMWP as well as protamine sulphate and its adduct (wherever applicable) in marketed insulin formulations and sequencing of protamine peptides through Electron Transfer Dissociation (ETD) with the help of state-of-the-art technique Size-exclusion chromatography (SEC) coupled with mass spectrometry (MS). The newly developed method offers an advantage owing to a combination of aqueous mobile phases leading to no particle shredding and online coupling of SEC to MS. Furthermore, reproducibility, high sensitivity, and enhanced resolution between insulin and HMWP as well as protamine sulphate and its adduct (wherever applicable) and short run time offer an added advantage to the method. Besides, the newly developed mass compatible SEC-MS method in combination to the ETD-MS/MS can provide direct information on species heterogeneity of protamine peptides.

2. Experiments

2.1. Instrumentation

Shimadzu UFLC coupled to Mass spectrometer LTQ Orbitrap (Thermo Scientific) XL and vanquish Thermo UPLC coupled with Thermo Scientific^{TM} - Orbitrap Lumos^{TM} MS were used in this study. Identification of HMWP species was carried out on Orbitrap XL while sequencing of protamine was carried out on Orbitrap Lumos with Electron transfer dissociation (ETD) capability. Separation of HMWP, protamine and insulin monomer was achieved by using Sepax column of 7.8*300 mm with particle size 3 μm with 300 Å pore size column at ambient temperature.

2.2. Chemicals and materials

All chemicals used were of analytical grade, different formulations of human insulin drug product like Humulin® R (soluble), Humulin® N (suspension), Humulin® 70/30 (mixed) with a label claim of 100IU/mL (10 mL Vials) were sourced from Eli Lilly, USA, with appropriate shipment and temperature control. Insulin analogs Novolog® prefilled 3 mL pen (Insulin Aspart-100IU/mL) procured from Novo Nordisk and Lantus® prefilled 3 mL pen (Insulin Glargine-100IU/mL) procured from Sanofi were used for assessing specificity of the method. All the samples used in the study were within expiry at the time of analysis.

2.3. Preparation of mobile phase and Samples

Mobile Phase: Methanol-0.1 % Difluroacetic acid (50:50, v/v) is used as a mobile phase. Thermally degraded samples were prepared by incubating Humulin® samples (10 mL Vials) at 60 °C for 3 days to accelerate HMWP formation.

2.4. Method

35 micrograms (100 IU/mL of Humulin® drug product; injection volume 10 μL) of thermally denatured sample was loaded onto Sepax column of 7.8*300 mm with particle size 3 μm with 300 Å pore size column at ambient temperature for in-line analysis on SEC-HPLC (Shimadzu UFLC) coupled to Mass spectrometer LTQ Orbitrap (Thermo Scientific). Column was equilibrated with isocratic flow of 0.5 mL/min with 20 % methanol for two column volumes. Elution was carried out using Methanol-0.1 % Difluroacetic acid (50:50, v/v) with flow rate of 0.5 ml/min for 45 min. The MS instrument was operated in positive mode with a capillary temperature of 300 °C, capillary voltage of 4 V and source offset of 40 V. Scans of 1 s were acquired from 700 to 4000 *m/z* for 40 min with

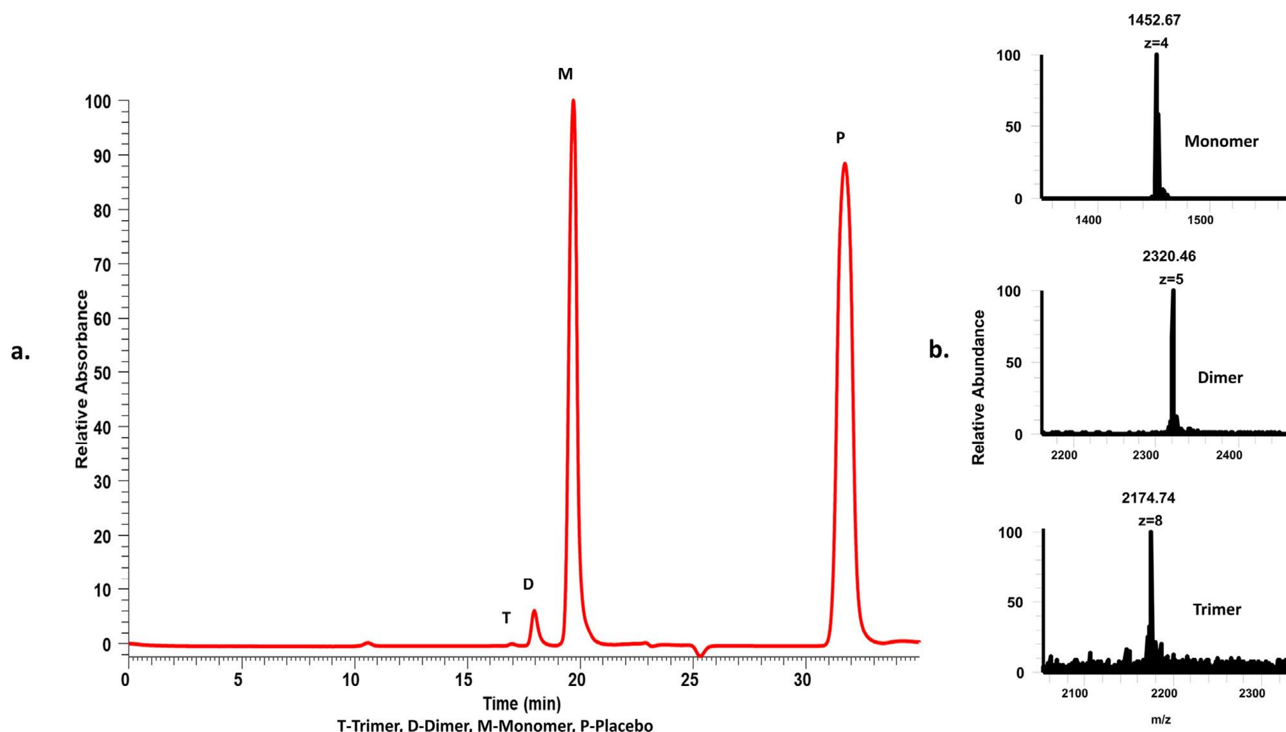

Fig. 1. a. UV chromatogram of Eli Lilly's Humulin® (Soluble formulation) incubated at 60 °C for 3 days, b. Corresponding mass spectra of HMWP and Monomer.

35.0 V collision induced energy as a one segment and 300−700 *m/z* as another segment to detect Protamine peptides.

For information on method development and validation, please refer supplementary material section

3. Results and discussion

3.1. Identification of HMWP by SEC-MS

Covalently linked aggregates of insulin formed because of chemical modification is referred to as High Molecular Weight Products (HMWP) and is quantitated through a pharmacopoeia assay based on Size exclusion chromatography (SEC) (USP43-NF38, Insulin human injection). The conventional method of size exclusion chromatography is modified by addition of mass spectrometer detectors making it productive for characterization of bio therapeutics. The advantages offered by this method are better separation, better peak shape, and most importantly online mass spectrometric analysis. Solvent based separation with organic modifiers and ion pairing reagents in buffer provides negligible secondary interactions making this method suitable for identification of HMWP species directly in mass spectrometer. To demonstrate the capability of the method, HMWP assessment was carried out on commercially available three formulations of Humulin® soluble, suspension and mixed (70/30). As a first step, Humulin® soluble formulation was incubated at 60 °C for 3 days and data acquired using SEC-MS in Thermo orbitrap XL with method conditions given in above section. UV profile was monitored at 214 nm instead of 276 nm as in the conventional SEC-UV method described in pharmacopeia. Change in detector wavelength was primarily done considering absence of aromatic amino acids in protamine sulfate, which is also assessed online in suspension formulations of Humulin®. As an illustration, UV chromatogram is shown in Fig. 1.

In Fig. 1 the elution order of peak 1–4 follows the elution behaviour of the Trimer, Dimer, Monomer and Placebo respectively. Mass to charge ratios of monomer (m/z-1452.67$_{(+4)}$), dimer (m/z-2320.46$_{(+5)}$), and trimer (m/z-2174.74$_{(+8)}$) shown in Fig. 1b. The delta mass for covalent insulin dimer is ∼−18 Da lesser than the mass of two insulin molecules, which is indicative of the peptide bond formation with a loss of one water molecule. The delta mass for covalent insulin trimer is ∼−36 Da lesser than the mass of three insulin molecules, where three insulin monomers connect to each other through peptide bond with loss of two water molecules. The resolution between monomer and dimer is 2.4, which is typically observed to negate any carry over effect.

Detecting Insulin dimers with different site modifications may not be possible in the presented method due to co-elution – but they are separable if a two-dimensional chromatography is employed with hydrophobic or ionic interactions in the second dimension (data not shown), which is then coupled to mass spectrometry. However, method demonstrates an online detection of covalently linked HMWP as well as insulin monomer in a simplistic manner.

It is known that Trifluroacetic acid (TFA) suppresses mass spectrometric signal, hence the method was developed with lower amount of Difluroacetic acid, which in combination with methanol gave reproducible results as shown in Fig. 2 delivering the method compatible for SEC-MS applications.

3.2. Identification and sequencing of protamine sulphate in insulin suspension formulation

In the SEC method, the covalent insulin dimer (CID) elute prior to the insulin monomer due to the larger molecular masses and hydrodynamic volumes, while preservatives elute later than the void volume of the column due to a weak hydrophobic interaction with the stationary phase. Despite a smaller molecular mass, protamine sulphate (PS) elutes prior to CID due to the relatively large hydrodynamic volume caused by the linear structure of the protamine peptide as mentioned in the Fig. 3 and corresponding mass spectra is shown in Fig. 4 [1].

The intensity of PS peak in N formulation is higher than 70/30 formulation, which is due to concentration difference of protamine used in the two formulations. [19]

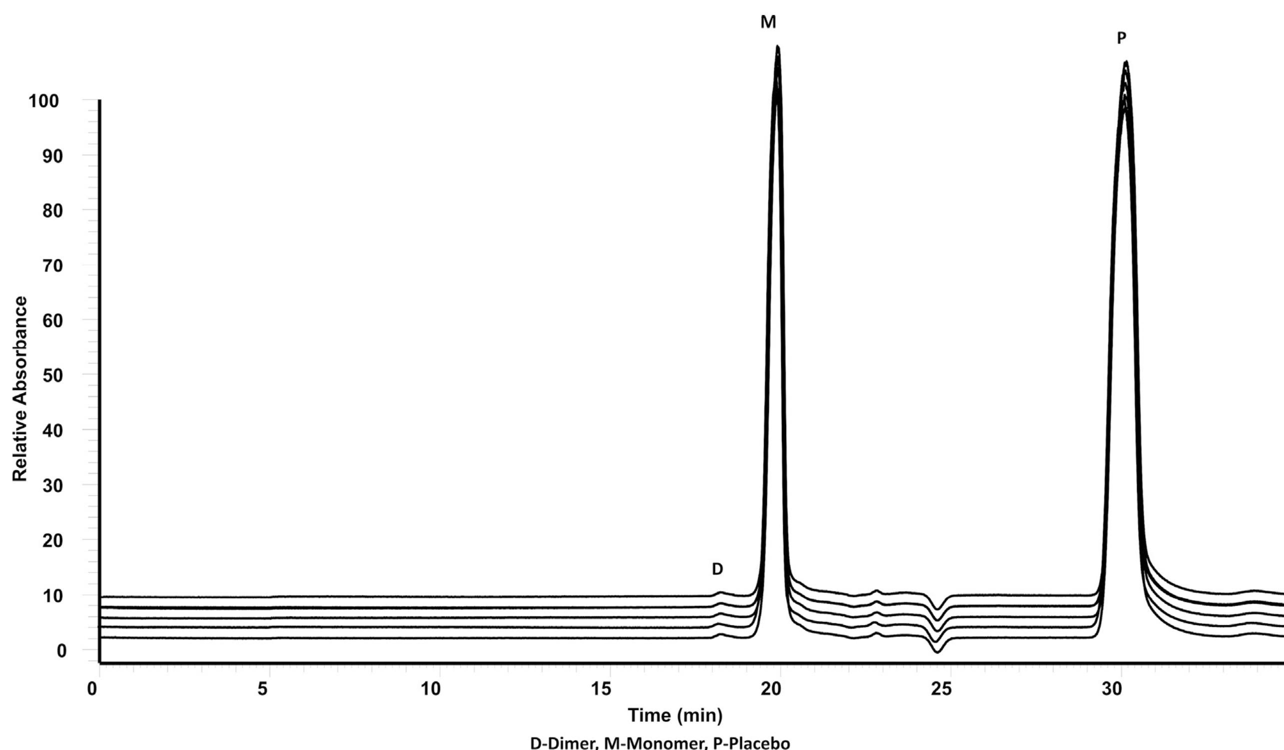

Fig. 2. UV chromatogram overlay six injections of Eli Lilly's Humulin® (Soluble formulation).

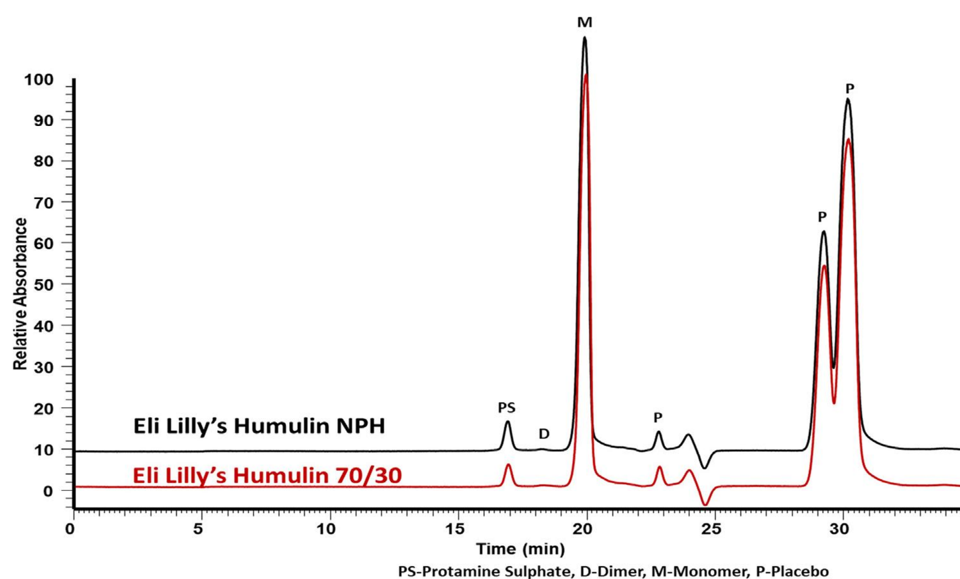

Fig. 3. UV chromatogram overlay of Eli Lilly's Humulin® N and 70/30 formulations.

Table 1
Protamine sulphate peptides along with the molecular weight.

US-Nomenclature	Sequence –US Reference	Molecular Weight (Average M+H) Da
Peptide-1	PRRRRRSSSRPIRRRRRPRASRRRRRGGRRRR	4236.45
Peptide-2	PRRRRSSRRPVRRRRRPRVSRRRRRRGGRRRR	4319.55
Peptide-3	PRRRRSSSRPVRRRRRPRVSRRRRRRGGRRRR	4250.45
Peptide-4	PRRRRASRRIRRRRRPRVSRRRRRGGRRRR	4064.40

Upon evaluation of mass spectra for PS peak, multiple peptides were observed out of which four dominant species (P_{1-4}) were identified as four peptides listed in USP standard certificate of analysis. Intact molecular weights of protamine peptides were tabulated in Table 1. Other than four major peptides, sulphate adducts (+98 Da) were observed that are labelled as P* in the spectra. By simultaneously detecting protamine sulphate and CID along with masses, this method acts as a powerful tool to assess the quality

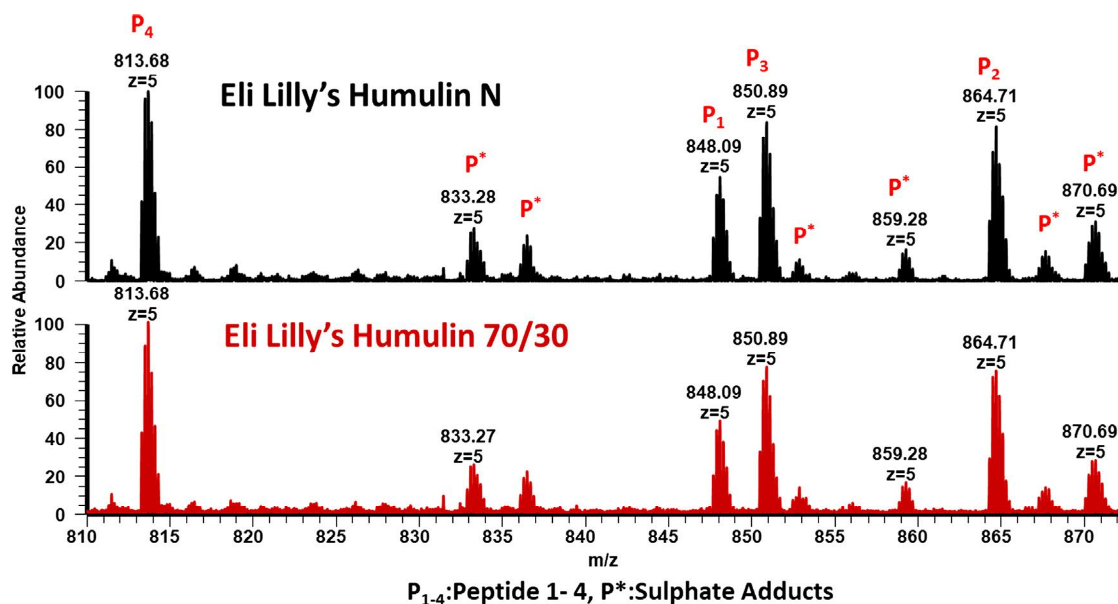

Fig. 4. Mass spectra of Eli Lilly's Humulin® N and 70/30 formulations.

Table 2
Theoretical Molecular Weights of CIPP.

Sample	Theoretical Molecular Weight in Da			CIPP Nomenclature
Protamine	Molecular weight	(Human Insulin protamine)	(Human Insulin protamine)-5Da	
Peptide-1	4236.93	10045.53	10040.53	CIPP-1
Peptide-2	4320.06	10128.66	10123.66	CIPP-2
Peptide-3	4250.95	10059.55	10054.55	CIPP-3
Peptide-4	4064.79	9873.39	9868.39	CIPP-4
Minor Species	4348.60	10157.20	10152.2	CIPP-5
Human Insulin	5808.6			

of product in two ways, one by demonstrating consistency in the heterogeneity of protamine sulfate peptides and second by characterizing CID species.

The MS/MS analysis of the protamine peptides was performed using Electron Transfer Dissociation (ETD) on the most abundant *m/z* values for each species [20]. Due to higher sensitivity and presence of basic residues (Arg), ETD *MSMS* was chosen over collisionally activated dissociation for sequencing of protamine peptides. ETD is an ion–ion reaction containing multiply charged macromolecule (proteins) cations and singly charged radical reagent anions. A free-radical-driven cleavage will undergo by the result of reagent anion transported to precursor cation. This cleavage dissociates NC—α bonds along the peptide backbone to generate even electron c-type and odd electron z•-type product ions that provide sequence information on the peptide precursor. In our experiment fluoranthene is used as ETD reagent for generating c- and z•-type ions. In ETD reaction mode the charge density of peptide precursors will play a crucial role in solving sequence [21]. For all tested samples, 99.9 % sequence coverage was obtained. A representative peptide map by ETD for the sequence coverage of the USP grade material of all four peptides is shown in Fig. 5.

3.3. *Identification of other higher order covalent species and insulin-protamine covalent product in mixed and biphasic formulated Insulin in force degraded product*

Human insulin samples of R, N and 70/30 formulations of Humulin® were thermally degraded at 60 °C for three days and were evaluated using SEC-MS method. In the degraded R formulation sample, a new aggregate species was identified along with the covalent insulin dimeric (CID) species. The species eluted prior to CID and upon mass evaluation it was identified as covalent insulin trimer (CIT).

No such trimeric species was observed in the heat stressed samples of N and 70/30 formulations. However, a new peak was observed prior to the protamine sulphate peak. Upon mass evaluation, the peak was verified as covalent insulin protamine product (CIPP), where the individual peptides of protamine were found to have formed a complex with a human Insulin molecule Table 2. The CIPP and the covalent insulin dimer (CID) elute prior to the insulin monomer due to the larger molecular masses and hydrodynamic volumes. Despite smaller molecular mass with respect to CID, CIPP elutes prior to CID due to the relatively large hydrodynamic volume caused by the linear structure of the protamine peptide moiety of the CIPP [22].

Increased formation of CIPP at 60 °C was observed for insulin drug products with higher amount of protamine, (N formulation: 0.35 mg/mL and 70/30 formulation: 0.24 mg/mL). This shows that the formation may increase with increase in temperature and content of protamine sulphate.

Ionization in the mass spectrometer of CIPP species in the force degraded Humulin® N and 70/30 samples was observed to be very poor. However, the Identity of the pre peak eluting before protamine peak in both N and 70/30 formulations can be termed as CIPP by correlating the masses found and reference from ***Beavis RC*** "A Novel Protein Cross-Linking Reaction in Stressed Neutral Protamine Hagedorn Formulations of Insulin" [23].

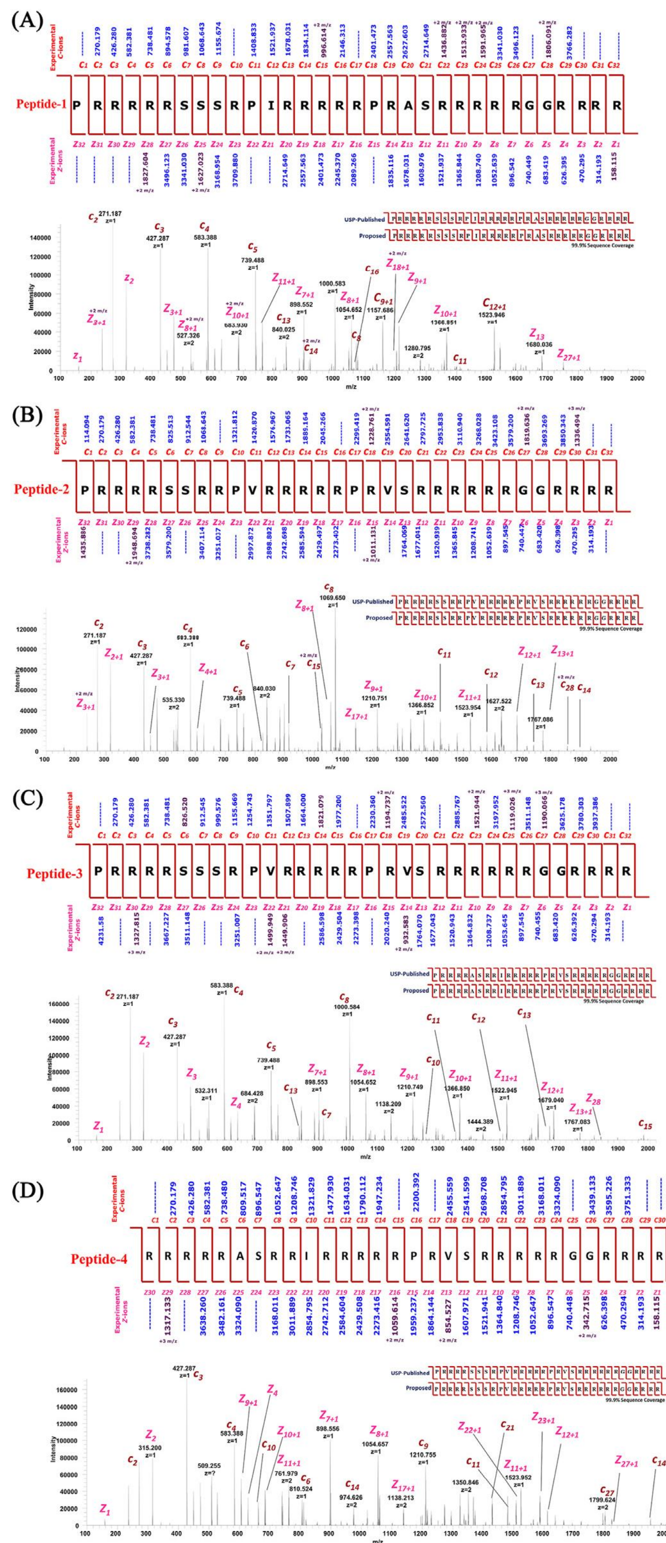

Fig. 5. ETD-MS/MS spectra for protamine Peptide-1 (a), Peptide-2 (b), Peptide-3 (c) Peptide-4 (D). Published and identified sequences (Identical) and fragment maps are inset in each spectrum.

3.4. Method applicability on different insulin analogs

Applicability of the method was evaluated by comparing the molecular weight of HMWP and monomer species in Insulin analogues (Insulin Glargine and Insulin Aspart), and Humulin®. Insulin analogs Novolog®, Lantus® and Humulin® showed different elution times and molecular masses for both monomer and HMWP species. Thus, the method was found to be specific for human insulin (and/or insulin analogues) and clearly assigns a molecular weight values for CID, monomer, and protamine sulphate. The

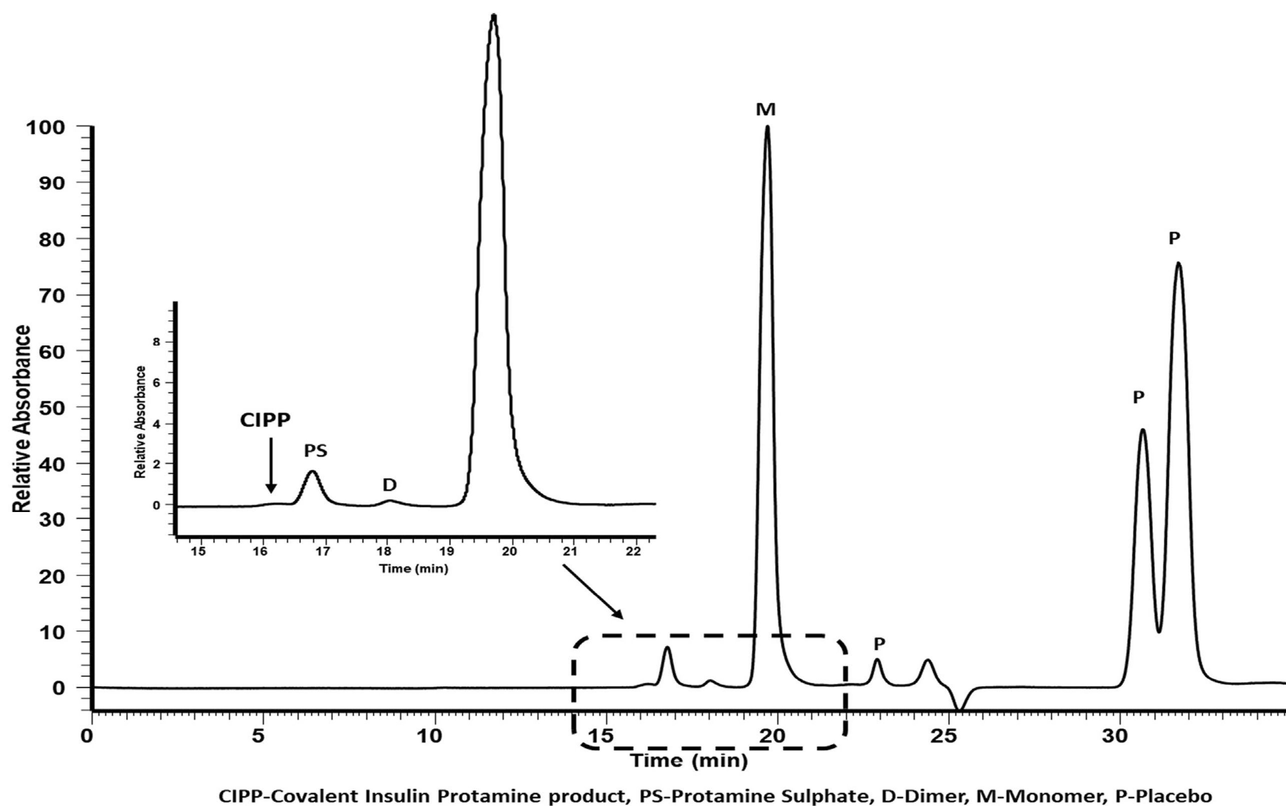

Fig. 6. UV chromatogram of Eli Lilly's Humulin® N thermally degraded at 60 °C for 3 days.

difference can be attributed to mass as well as elution pattern Fig. 6.

4. Conclusion

We have demonstrated a newly developed SEC-MS method for qualitative characterization of covalent HMWP, protamine peptides, and covalent insulin protamine product present in recombinant insulin (as well as analogues) formulations. Currently used RP-HPLC method to quantify protamine peptides employs mobile phases with high salt content. Such a method can't be coupled to MS. Modified mobile phase system with MS compatible solvents, may still not be beneficial owing to high hydrophilicity of the protamine peptides leading to elute in the void region (or near void region). Such an elution along with salts present in the sample may supress the ionisation, which makes researchers very hard to assign ions after fragmentation (ETD). Alternatively, a direct infusion of protamine peptides in mass spectrometer would require extensive sample preparation as well as more caution and expertise in handling MS. This method allows accurate identification of variants of protamine peptides by sequencing through ETD in an online manner. The method made use of conventional methodology to identify covalent HMWP in different insulin analogues and state-of-the-art mass spectroscopy tools including ETD-MS/MS. Method set up is simple, requires low sample volumes, ease in mobile phase preparation and due to the mass compatible mobile phase provides a direct access to mass spectrometer. The additional advantage of sequencing of protamine peptides by ETD-MS/MS allow users to characterize and confirm protamine species and helps in selecting closely matched lot with USP standard for formulating the biphasic and mixed formulations of human insulin. The method has a potential to be a very handy tool especially for demonstrating comparability between a reference product and a biosimilar product.

Author contributions

KS: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Original draft; YG: Data curation, Formal analysis; PPC: Supervision, validation, review, and editing; AK: Supervision, review, project administration, and editing; NV: Supervision, review, and editing, project administration, Resources, Methodology

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2021.114188.

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