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Original Article

Comparison of Two Pharmacopoeia Methods for Determining Higher Molecular Weight Proteins in Insulin Glargine

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ABSTRACT

Objective: We compared two pharmacopoeia methods, European Pharmacopoeia (EP) and Indian Pharmacopoeia (IP),with respect to their key characteristics for determining higher molecular weight proteins (HMWP) in insulin glargine. These methods differ in the mobile phase composition, number of HPLC columns used and run time. **Material and Methods:** Testing was carried out exactly as described in the respective pharmacopoeia methods. **Results:** Our results indicate that both methods provide insulin glargine peak with comparable symmetry factor. Results obtained with two methods are precise in terms of intra-assay variation; however, inter-assay variation was better with IP method. Also, both methods provide similar results in terms of estimation of HMWP content in insulin glargine. **Conclusion:** Based on the findings we propose that IP method may replace EP method for determination of HMWP content in insulin glargine which will reduce the analysis time and running cost of the test without compromising with the results.

Key words: HMWP, Insulin glargine, Monomer, Pharmacopoeia, Size exclusion HPLC.

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INTRODUCTION

The advent of recombinant DNA (rDNA) technology and its application in the pharmaceutical industry has brought about a rapid growth in the development of a number of therapeutic rDNA products for human use. Human insulin produced by rDNA technology is the first commercial health care product derived from this technology.[1] Utilizing various genetic engineering techniques, pharmaceutical companies have developed a variety of rapid-or long-acting insulin analogues providing more choices for diabetes patients. All insulin analogues are modified from the insulin human gene using genetic engineering techniques to produce amino acid substitutions resulting either decrease or increase in the absorption time from the subcutaneous tissue with corresponding changes in time-action profile.[2] Among the insulin analogs, insulin glargine has been developed as a basal insulin which shows constant release from the site of injection with no pronounced peak. Insulin glargine differs from insulin human in that the amino acid AsnA21 is replaced by glycine and 2 arginines are added to the C-terminus of the B-chain.[2]

Quality evaluation for critical quality attributes of insulin glargine is carried out in compliance with the requirements of European pharmacopoeia (EP).[3] As per current pharmacopoeia requirements, impurities of higher molecular weight proteins (HMWP) of insulin glargine are determined by size exclusion HPLC (SE-HPLC) using two HPLC colums in series with run time of about 65 min.[3] In our work we focused on Indian pharmacopoeia (IP) method for determining HMWP of insulin glargine. IP method is already a well established method based on using single HPLC column with run time of approximately 35 min[4] and is employed for estimating HMWP in human insulin, insulin aspart and insulin lispro.[4-6] As the molecular weight and amino acid sequence of insulin glargine and human insulin are very similar, we expect that the two molecules should have similar chromatographic profiles when tested for their HMWP content by SE-HPLC. Also, it would be interesting to investigate the results of test on HMWP of insulin glargine by the method described in IP. Therefore, we planned this study to compare two SE-HPLC based pharmacopoeia methods, *i.e.* EP and IP, for the determination of HMWP in insulin glargine.

MATERIALS AND METHODS

Instrumentation

HPLC analyses were performed on an Agilent HPLC 1260 Infinity system (Agilent Technologies, Germany) fitted with G1311C pump, G1319B auto sampler and G1315D diode array detector (DAD). Experiments were carried out as per EP method using two HPLC columns coupled in series (Shodex Protein KW 802.5, 7.8×300 mm) and as per IP method using single HPLC column (Waters Insulin HMWP column, 7.8 × 300 mm).

Chemicals and Solvents

HPLC-grade organic solvents were purchased from Rankem (Faridabad, India). L-arginine was purchased from Loba Chemie (Mumbai, India). HPLC grade water was prepared using Milli-Q water purification system (Millipore, Bangalore, India). Insulin glargine drug substance and insulin glargine reference standard were courtesy donations from Sanofi India Ltd., Mumbai.

Reference solution preparation

Insulin glargine reference standard was allowed to dry in an oven (Steris, Canada) at 100°C for 2 h. Approximately 15 mg of dry insulin glargine powder was dissolved in 1.5 ml of 0.01N HCl and diluted to 10 ml in a volumetric flask with water to prepare reference solution.

Chromatographic conditions

Mobile phases were prepared for two HPLC methods as described in respective pharmacopoeia monographs.[3,4] Briefly, mobile phase for EP method was prepared as a mixture of 20 volumes of glacial acetic acid,

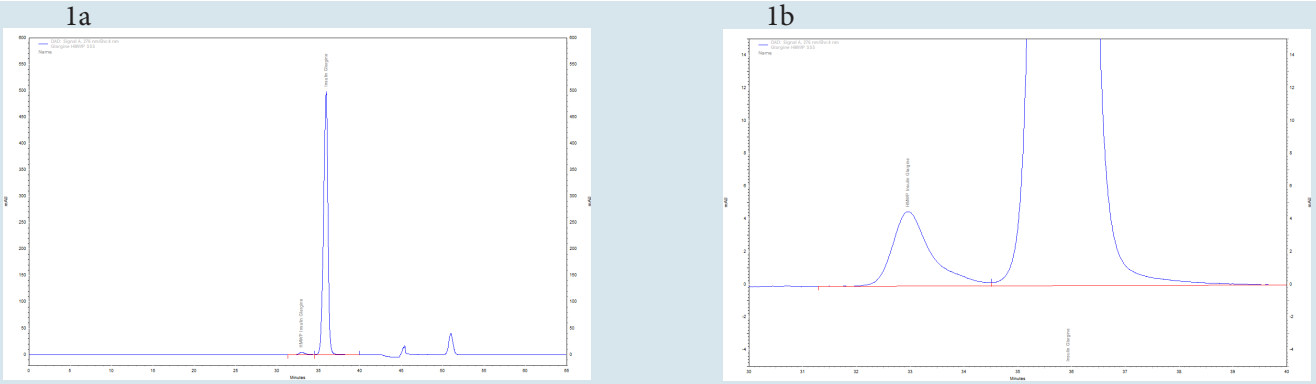

Figure 1: Chromatogram of reference solution obtained with EP method 1(a) and its enlarged view 1(b).

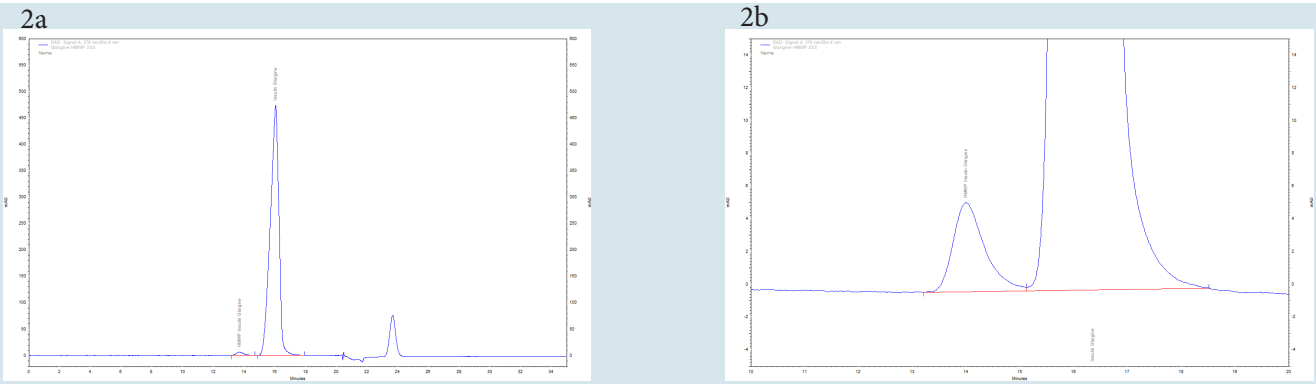

Figure 2: Chromatogram of reference solution obtained with IP method 2(a) and its enlarged view 2(b).

30 volumes of acetonitrile, 50 volumes of water and pH was adjusted to 3.0 with ammonia solution. Similarly, mobile phase for IP method was prepared by mixing 65 volumes of 0.1% (w/v) solution of L-arginine in water, 20 volumes of acetonitrile and 15 volumes of glacial acetic acid. Mobile phases were filtered through 0.45-mm nylon filter (Pall Life Sciences, Mumbai, India) and degassed using ultrasonic bath sonicator for 30 min before running the experiment. All experiments conducted on the HPLC were carried out in isocratic mode. Injection volume was 100 µL with a flow rate of 0.5 mL/min. The column temperature was maintained as ambient and elution was monitored at 276 nm using the DAD detector. Run time for EP and IP methods were 65 min. and 35 min. respectively. Data acquisition and processing was done with EzChrom Elite software.

Test validity

Tests were considered valid when symmetry factor was maximum 2.0 for insulin glargine peak and peak to valley ratio was minimum 2 in the reference solution.[3]

RESULTS AND DISCUSSION

In present study, two SE-HPLC methods were performed for determination of HMWP content of insulin glargine. In SE-HPLC components of a mixture are separated according to their molecular size based on the flow of the sample through the porous packing material. Large molecules (eg. HMWP) that cannot penetrate the pores of the packing material elute first from the column and smaller molecules (eg. insulin glargine monomer) can partially or completely enter the packing material and elute from the column later. The EP method recommends SE-HPLC of insulin glargine using a mobile phase containing water, glacial acetic acid, and acetonitrile (pH adjusted to 3.0) for 65 min using two HPLC columns coupled in series. Stationary phase described in EP monograph states use of hydrophilic silica gel (5 µm) with a pore size of 15 nm of a grade suitable for fractionation of globular proteins in the relative molecular mass range of 2000 to 80000. With this method retention time of insulin glargine monomer peak is expected to be approximately 35 min.[3] In contrast IP method recommends SE-HPLC for determination of HMWP in human insulin using a mobile phase of L-arginine in water, glacial acetic acid, and acetonitrile for approximately 35 min using single HPLC column. IP method describes stationary phase as hydrophilic silica gel (5-10 µm) of a grade suitable for separation of insulin monomer from dimer and polymer. With this method the retention time of insulin peak is approximately 17 min.[4]

Representative chromatograms of the reference solution obtained with two methods are shown in Figure 1 and 2. In the present study retention time of insulin glargine monomer peak with EP method was 36.2 ± 0.46 min (n=9) and average retention time of insulin glargine HMWP peak was 32.6 ± 0.44 min. When insulin glargine samples were analysed for HMWP as per IP method, average retention time of insulin glargine peak and insulin glargine HMWP peak were 16.5 ± 0.64 min (n=9) and 14.07 ± 0.64 min respectively. Results obtained in the present study show that when insulin glargine is tested with two pharmacopoeia methods for HMWP, retention times of the insulin glargine monomer and HMWP peaks are as per the pharmacopoeia requirements. These observations confirm our hypothesis that chromatographic profiles of insulin glargine

should match with that of human insulin in SE-HPLC. The variation observed for the retention time of the principal peak in two methods is attributed to the difference in column length. EP method employs two columns (300 mm each) in series making total column length equivalent to 600 mm resulting in increased retention time of approximately 36 min. In contrast, IP method which utilizes a column of 300 mm length gives retention time of approximately 16.5 min.

Estimation of symmetry factor of the peak is one of the important parameter of the HPLC analysis. Results of our study show that average symmetry factor of the principal peak in reference solution was 0.80 and 0.82 for EP and IP methods respectively indicating that two methods do not show any significant difference for the peak symmetry. The EP monograph on insulin glargine mentions that for the validity of the test for HMWP, the symmetry factor of the principal peak should not be more than 2. In this regard, results of both methods comply with the current pharmacopoeia requirements.[3] Also, another important test validity parameter mentioned in EP monograph is peak to valley ratio. Maximum peak to valley ratio obtained in the present study was 12 and 20 for EP and IP methods respectively indicating that IP method may offer an advantage with respect to this validity parameter.

Chromatographic principles predict that resolution between two peaks is proportional to the column length and that is what has been observed in the present study. Average resolution between HMWP and insulin glargine monomer peak was 2.61 and 2.11 for EP and IP methods respectively. Improved resolution with EP method is attributed to the increased column length. Although use of two HPLC columns in EP method results in better resolution between HMWP and monomer peaks, this improved resolution is accompanied by almost two-fold increase in analysis time and mobile phase use. It is important to mention here that resolution is currently not a pharmacopoeia requirement for the test on HMWP.

In our study, intra-assay and inter-assay variations in the determination of HMWP content with two methods were assessed by taking into consideration the %peak area of the HMWP peak obtained with reference solution. For the calculation of intra-assay variation, reference solution was injected three times and %RSD of the HMWP content were calculated. Results show that for EP and IP methods the intra-assay variation was 0.50% and 0.55% respectively. These results indicate that both EP and IP methods are highly precise in terms of intra-assay variation without any significant difference. Inter-assay variation was also assessed by taking into consideration the % area of the HMWP peak obtained with reference solution from three independent experiments. Our results show that inter-assay variation was 16.25% for EP method and 7.42% for IP method. These results indicate that IP method is comparatively more precise in terms of inter-assay variation. Two methods were also compared with each other to evaluate their ability to estimate HMWP content in insulin glargine. Present study show that estimated mean HMWP content in reference solution was 1.32% and 1.15% when tested by EP and IP methods respectively. This indicates that the two methods are comparable with respect to estimating HMWP content in insulin glargine.

An impurity is defined as any component present in the drug substance or drug product which is not the desired product and it may be either product-related or process-related. HMWP present in drug substance or drug product is considered as an impurity. Various factors such as impact of heat, light, acids, bases, changes in the pH of the formulation and interactions with packaging components may contribute in generation of HMWP. Identification, quantification and control of impurities in the drug substance and drug productare an important part of drug development and regulatory quality assessment.

CONCLUSION

Overall, the results of present study indicate that both EP and IP methods provide comparable results for the determination of HMWP in insulin glargine and EP method does not offer any advantage over the IP method. In addition, EP method is more time consuming and gives final results in approximately 65 min. The longer analysis time and use of two HPLC columns result in significant increase in the mobile phase consumption and running cost of the test. In view of these results, it is proposed that IP method, which gives results in approximately 35 min, may be considered as a method of choice for estimation of HMWP content in insulin glargine and may replace the EP method.

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CONFLICT OF INTEREST

The author has no conflict of interest.

ABBREVIATION USED

DAD: Diode array detector; **EP:** European pharmacopoeia; **HMWP:** Higher molecular weight proteins; **IP:** Indian Pharmacopoeia; **rDNA:** Recombinant DNA; **SE-HPLC:** Size exclusion HPLC.

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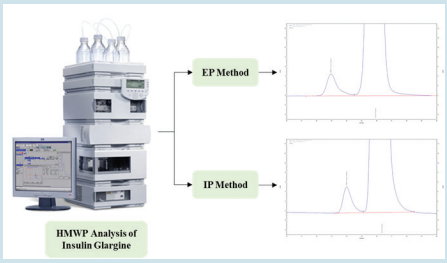
SUMMARY

- EP and IP methods provide Insulin glargine peak with comparable tailing factor.Results with two methods are precise in terms of intra-assay variation and inter-assay variation was better with IP method.
- Both methods are comparable in terms of estimation of HMWP content in Insulin glargine without showing any major difference.
- IP method may replace EP method for determination of HMWP content in Insulin glargine.

PICTORIAL ABSTRACT



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