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Identification of Recombinant Insulin Analogues by Peptide Mapping Method

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ABSTRACT

Insulin human, containing 51 amino acids, is a small polypeptide hormone that regulates blood glucose homeostasis. Patients with insulin-dependent diabetes mellitus require insulin therapy through the administration of exogenous insulin to avoid ketoacidosis. Utilizing various genetic engineering techniques, pharmaceutical companies have developed a variety of rapid- or long-acting insulin analogues. Analytical methods for various types of insulin and insulin analogues are gradually being included in the United States Pharmacopeia (USP) and the European Pharmacopeia (Ph. Eur.), but not yet in the Chinese Pharmacopeia. Usually these insulin analogues only differ by 1 to 3 amino acids, which is too subtle to distinguish by most of analytical methods currently available. In this study a peptide mapping technique was employed to screen insulin analogues for quality assessment. Peptide mapping is capable of identifying single amino acid changes resulted from events such as errors in the reading of complementary DNA sequences or point mutations. Here we analyzed 6 insulin preparations including insulin human, insulin lispro, insulin aspart, insulin detemir, insulin glargine and insulin glulisine using peptide mapping analysis. The peptide fingerprints of the insulin products we tested all corresponded well to those of the standard materials. Our peptide mapping method is more accurate in identifying the subtle differences between the insulin analogues than chromatography is. We concluded that peptide mapping is a valuable initial screening tool for quality assessment of insulin analogues. In the future, we intend to continue to develop this technology for post-marketing surveillance of other biopharmaceuticals and biosimilars, such as somatropin, erythropoietin and G-CSF.

Key words: insulin, insulin analogues, peptide mapping, high performance liquid chromatography

INTRODUCTION

Human Insulin is produced by the beta cells of the pancreas, which are located in the islets of Langerhans[1]. Although insulin is active as a monomer, it assembles to dimmers and hexamers in the presence of zinc during biosynthesis and storage[2]. Because of its evolutionarily conserved gene structure across species, insulin extracted from bovine and porcine pancreas is given to people with diabetes mellitus in early insulin therapy[3]. Banting and Best extracted bovine insulin in 1921 and successfully administered it to patients with diabetes mellitus[4]. After undergoing evolutionary manufacturing, recombinant insulin was the first approved biotechnology-derived drug product in 1982. Recombinant insulin has subsequently replaced purified insulin from animal sources in clinical therapy. Within 15 years, a further 5 insulin analogues have been developed by pharmaceutical companies providing more choices for diabetes patients (Table 1). All insulin analogues are modified from the insulin human gene using genetic engineering techniques and produced in *E. coli* or yeast. Amino acid substitution in insulin lispro, insulin aspart, and insulin glulisine allows a more rapid onset of action and shorter duration of activity. Long-acting basal analogues such as insulin glargine and insulin detemir have a slower onset of action and a longer duration of action[5,6]. Depending on disease progression, optional insulin therapy can reduce injection frequency and side effects, and thus improve quality of life[7].

Insulin human is a 2-chain polypeptide hormone composed of 51 amino acids with a molecular weight of 5,808 Daltons (Figure 1A). The A-chain is composed of 21 amino acids and the B-chain is composed of 30 amino acids. The 3-dimensional structure of insulin is further stabilized by disulfide bridges between thiol groups (-SH) on cysteine residues. Insulin has 6 cysteines that form 3 disulfide bridges: 2 form an interchain between the A and B chains (between A7 and B7, and A20 and B19), and 1 forms an intrachain within the A-chain (between A6 and A11)[8,9]. Insulin lispro

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Table 1. Comparison of insulin human and insulin analogues

Name	insulin human	insulin lispro	insulin aspart	insulin glargine	insulin glulisine	insulin detemir
MW(Da)	5808	5808	5825.8	6063	5823	5916.9
Acting	short	rapid	rapid	long	rapid	long
Host cell	*E. coli*	*E. coli*	yeast	*E. coli*	*E. coli*	yeast
Approval year	1982	1996	2000	2003	2004	2006
Modification	–	Pro^{B28}→Lys^{B28} Lys^{B29}→Pro^{B29}	Pro^{B28}→Asp^{B28}	Asn^{A21}→Gly^{A21} + $Arg^{B30\text{-}B31}$	Asn^{B3}→Lys^{B3} Lys^{B29}→Glu^{B29}	Omit Thr^{B30} + C14-FA^{B29}

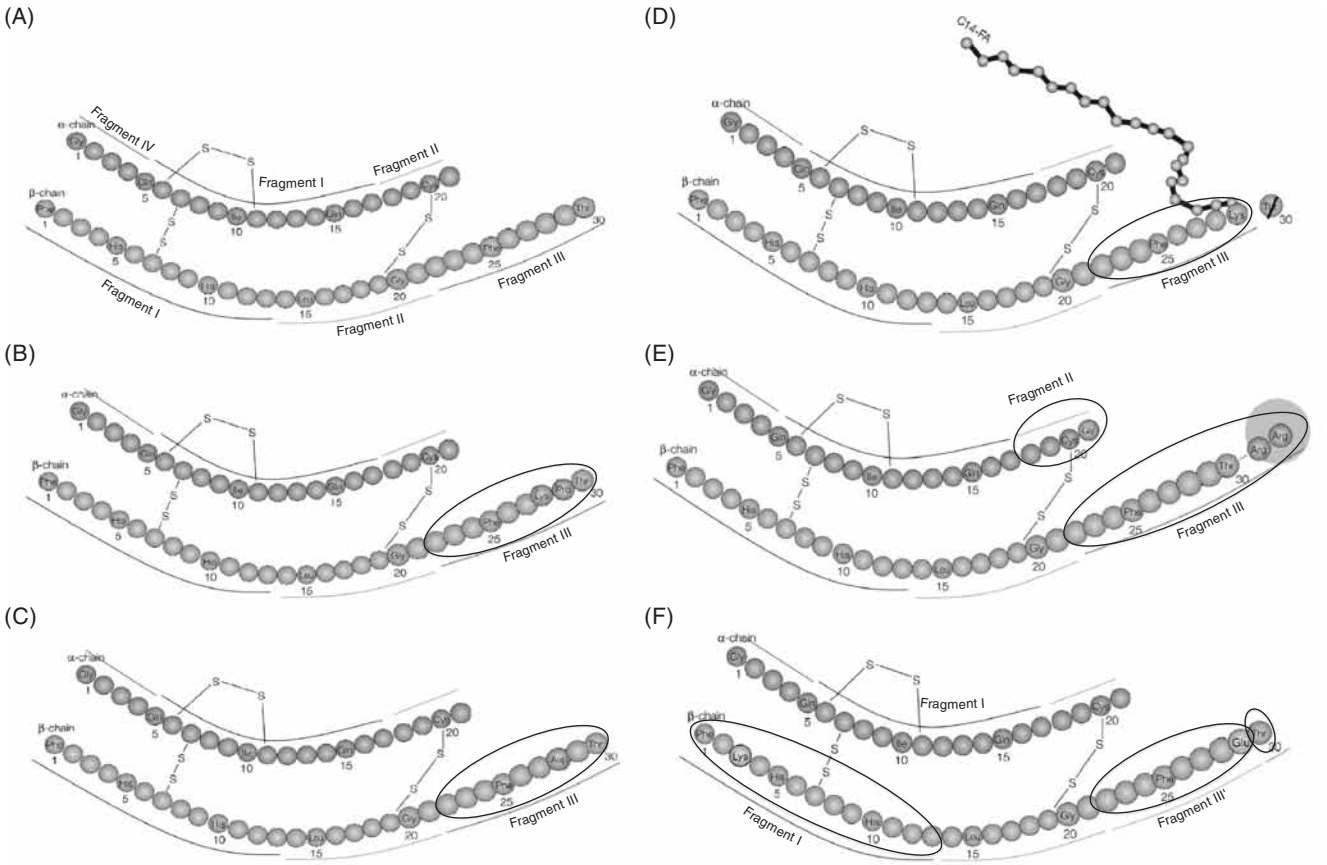

Figure 1. Diagrams of structures and Glu-C proteinase V8 digestion sites of (A) insulin human, (B) insulin lispro, (C) insulin aspart, (D) insulin detemir, (E) insulin glargine, and (F) insulin glulisine. Peptide fragments generated from protease V8 treatment were labeled as I, II, III, and IV. The modification sites of insulin analogues are circled by dotted lines.

is identical in primary structure to insulin human, only differing in amino acid sequence at positions 28 and 29 of the B-chain. Insulin human is Pro^{B28}, Lys^{B29}, whereas insulin lispro is Lys^{B28}, Pro^{B29} (Figure 1B). As it has the same amino acid composition, the molecular weight and charge value of insulin lispro are the same as insulin human[10,11]. Insulin aspart has Asp^{B28} instead of Pro^{B28} (Figure 1C)[12]. Because of the change in amino acid composition, the molecular weight of insulin aspart is slightly greater than that of insulin human at 5,825.8 Daltons. Insulin detemir is a long-acting insulin analogue in which a myristic acid is bound to the Lys^{B29} and it has a molecular weight of 5,916.9 Daltons[13] (Figure 1D). Insulin glargine differs from insulin human in that the amino acid Asn^{A21} is replaced by glycine and 2 arginines are added to the C-terminus of the B-chain (Figure 1E)[14]. It has a molecular weight of 6,063 Daltons and an isoelectric point of 6.7, which contrasts with the native insulin human, which has an isoelectric pH of 5.4. The rapid acting profile of insulin glulisine is achieved by substitution of 2 amino acids in the B-chain of insulin human, where Asn^{B3} is replaced by Lys and Lys^{B29} is replaced by Glu (Figure 1F)[15]. The amino acid substitutions in insulin glulisine destabilise the hexamers and therefore enable a faster onset of action than that achieved with insulin human. The molecular mass of insulin glulisine is 5,923 Daltons.

Since the insulin detemir, insulin glargine and insulin

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glulisine are not included in the pharmacopeia of various countries, we consulted all the insulin-related monographs in Ph. Eur. and USP to set up a standardized analytical method which is available for their identification. In the Ph. Eur. monographs the identification of the insulin human, lispro, and aspart consists of a combination of 2 analytical methods, the liquid chromatograms and peptide mapping. In the USP monographs of insulin human and lispro the same approach is used. In this study we set up a peptide mapping method suitable for screening insulin human and all insulin analogues. Peptide mapping is a method of identifying proteins, especially those obtained by recombinant DNA technology. It is a powerful test capable of identifying single amino acid changes resulted from events such as errors in point mutations and can be used to evaluate the stability of the expression construct of cells used for recombinant DNA products and the consistency of the overall process, and to assess product stability, as well as to ensure the identity of the protein product, or to detect the presence of protein variants [16,17]. We performed peptide mapping to evaluate the quality of commercial insulin products, as well as a routine method for post-marketing surveillance.

MATERIALS AND METHODS

I. *Materials and Reagents*

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Thermo Fisher Scientific HyClone. HPLC grade acetonitrile, sequencing grade endoproteinase Glu-C from *S. aureus* V8, ammonium sulfate ($(NH_4)_2SO_4$), hydrochloride (HCl), 3-methylphenol (m-cresol), phenol, and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO). The Ph. Eur. reference standards of insulin human (batch number 3), insulin lispro (batch number 1), and insulin aspart (batch number 1) were purchased from the European Directorate for the Quality of Medicines & HealthCare (EDQM). The in-house working standards were obtained from Eli Lilly (insulin human and insulin lispro; Indianapolis, IN), Novo Nordisk (insulin aspart and insulin detemir; Princeton, NJ), and Sanofi-Aventis (insulin glargine and insulin glulisine). The commercial products Humulin, in which the active substance is insulin human, and Humalog, in which active substance is insulin lispro, were obtained from Eli Lilly. The commercial products NovoRapid, in which the active substance is insulin aspart, and Levemir, in which the active substance is insulin detemir, were obtained from Novo Nordisk. Milli-Q grade water (Millipore, Milford, MA) was used for buffer preparation and sample dilution. The Amicon Ultra-0.5 3K Centrifugal Filter unit was purchased from Millipore.

II. *Enzymatic Digestion*

Enzymatic digestion was performed according to the procedure described in monograph of insulin human in the European Pharmacopeia, with minor modifications. Briefly, the digestion was carried out by diluting the insulin solution to 2.0 mg/mL with 0.01 M HCl and then transferring 170 µL of this solution to a new tube. Six hundred and eighty microliter of incubation buffer, 0.1 M HEPES, pH 7.5 adjusted with 5 M NaOH, was added to the insulin solution. The digestion was initiated by addition 136 µL *S. aureus* V8 endoproteinase, which was prepared in water with a concentration of 1 mg/mL. The digestion was carried out for 6 h at 25°C and then quenched with 986 µL of sulfate buffer, which composed of equal volumes of 2.0 M $(NH_4)_2SO_4$ and 0.5 M sulfuric acid. The final concentration of insulin in the digested sample solution was about 172 µg/mL. To investigate the alteration of the excipients in the process of enzyme digestion, Amicon Ultra-0.5 3K Centrifugal Units were used to remove most of the preservatives and stabilizer in the formulation of insulin products prior to the enzyme digestion. The digested protein samples were analyzed immediately or stored at 4°C for analysis within 48 h.

III. *Reverse-Phase High Performance Liquid Chromatography*

RP-HPLC was performed on a Dionex UltiMate 3000 HPLC system with a Diode Array Detector (USA). A 2-eluent linear gradient system was employed with a flow rate of 0.8 mL/min. The mobile phase included water with 10% acetonitrile and 20% sulfate buffer as eluent A, and water with 40% acetonitrile and 20% sulfate buffer as eluent B. The initial condition was 5% eluent B for 3 min, and then 5-59% eluent B from 3 to 30 min, 59-80% eluent B from 30 to 35 min, 80-5% eluent B from 35 to 40 min, and 5% eluent B left for further 10 min to re-equilibrate. Fifty microliter digested sample solutions were injected and analyzed on an Inertsil ODS-3 C18 column (4.6×100 mm, 3 µm particle size; GL Sciences, Japan) maintained at a column temperature of 40°C. Fractionated insulin and insulin analogue peptides were detected by UV absorbance at 214 nm and compared to the reference standards. Peak areas were calculated using Chromeleon software (Dionex, USA).

RESULTS

First of all, the HPLC chromatograms of the intact insulin human and insulin analogues were examined. The major peaks of undigested insulin human and insulin analogues were approximately overlapped indicating a high degree of similarity between these molecules (Figure 2). In addition, undigested insulin detemir could not be detected under our standard analytical condition (Figure 2D). After that, peptide mapping using a Glu-C proteinase for digestion and subsequent separation of the fragments by HPLC was employed. To eliminate interference from these preservatives, a centrifugal filter was used to exclude molecules smaller than 3,000 Daltons before enzyme treatment. Although it was impossible to remove all the meta-cresol or phenol, filtering did

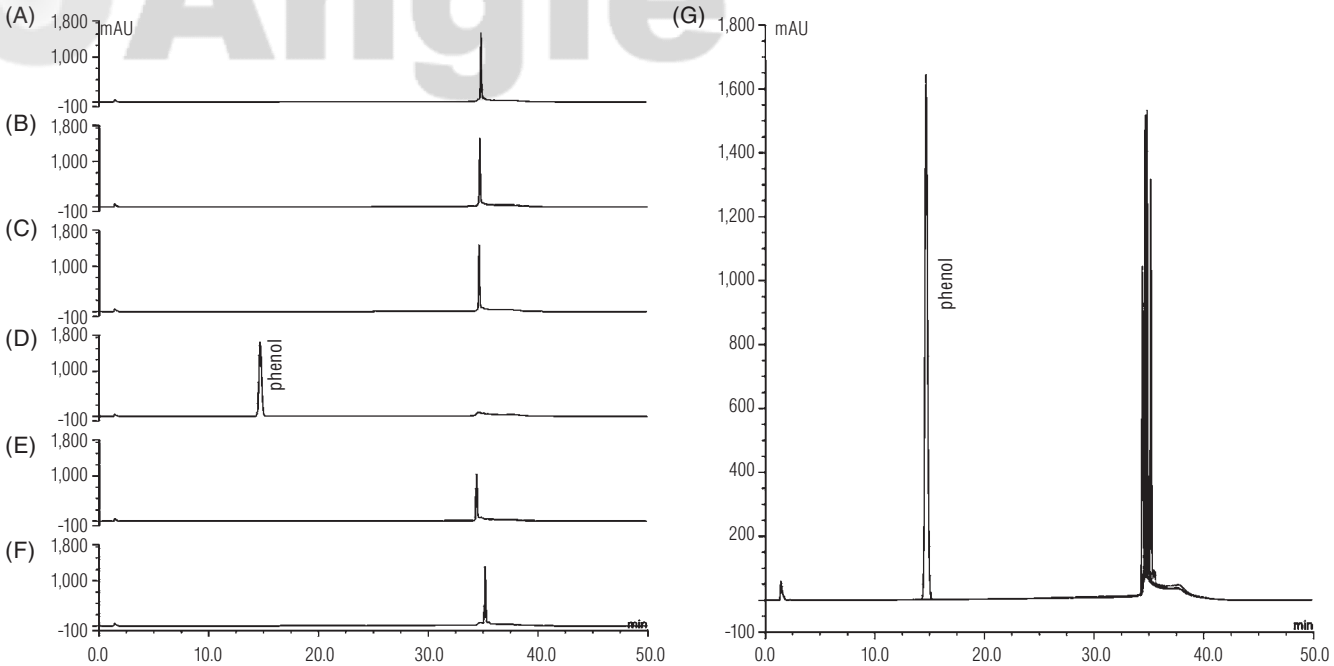

Figure 2. Chromatograms of undigested Ph. Eur. reference standards (A) insulin human, (B) insulin lispro, (C) insulin aspart, and of undigested in-house standards (D) insulin detemir, (E) insulin glargine, (F) insulin glulisine. (G) Chromatogram of undigested insulin mixture.

lower the amount of preservatives considerably and did not influence the retention time of the peptide fragments (data not shown). Overall, since the preservatives did not affect the enzyme activity, they were not removed to reduce artificial errors in subsequent experiments.

Four peptide fragments were generated from insulin human after protease V8 digestion and labeled as Fragment I, II, III, and IV according to their molecular weight from high to low (Figure 3A). Fragment I contained amino acids A5-A17 and B1-B13, Fragment II A18-A21 and B14-B21, Fragment III B22-B30, and Fragment IV A1-A4. These 4 peptide fragments eluted sequentially with the smaller peptide fragments eluting more rapidly. Fragment IV of 4 amino acids eluted first in less than 5 min and showed the lowest UV absorbance. Fragment III of 9 amino acids eluted at about 21.4 min followed by Fragment II, which contained 12 amino acids, eluted from the column about 1.5 min later than Fragment III. The UV absorbance of Fragment III was detected at a similar level to that of Fragment II. Fragment I containing 26 amino acids eluted at about 32 min and showed the highest level of UV absorption.

Except for insulin glulisine, the amino acid modifications on insulin analogues do not alter the cleavage site of Glu-C proteinase or the disulfide bonds, enzyme digested insulin analogues produces a similar peptide map to insulin human. A comparison of the peptide map of insulin lispro and insulin human, revealed a remarkable shift in the retention time of Fragment III that did not exist in the other 3 fragments (Figure 3B). Fragment III of insulin lispro (Lys^{B28}-Pro^{B29}) was eluted 2 min earlier than that of insulin human (Pro^{B28}-Lys^{B29}). A similar phenomenon was revealed on the peptide

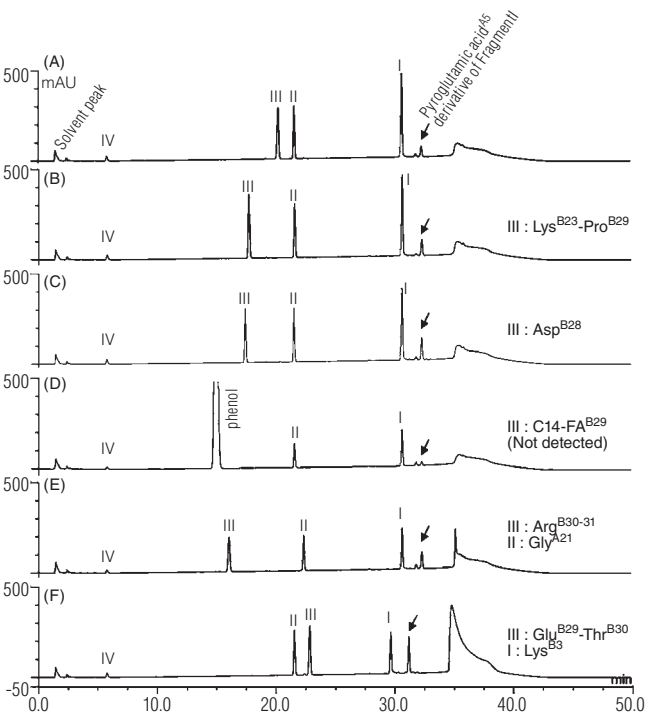

Figure 3. Peptide mapping of (A) insulin human, (B) insulin lispro, (C) insulin aspart, (D) insulin detemir, (E) insulin glargine, and (F) insulin glulisine. Peptide fragments of insulin analogues different from those of insulin human are indicated on the right side. Black arrows mark pyroglutamic acid^{A5} derived from Fragment I.

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maps of insulin aspart, where Fragment III of insulin aspart (Asp^{B28}) was eluted 2 min earlier than that of insulin human (Pro^{B28}) (Figure 3C). The peptide maps of insulin detemir showed that Fragments I, II, and IV having identical retention time to those of insulin human except Fragment III, which was not detected in chromatogram (Figure 3D).

Insulin glargine showed a significant shift in the retention times of fragments II and III as compared with those of insulin human (Figure 3E). Substitution of Asn^{A21} with Gly on insulin glargine delayed the retention time of Fragment II. Moreover, addition of 2 arginines on Fragment III of insulin glargine (Arg^{B31-32}) brought the elution ahead of that of insulin human. Peptide Fragments II and IV of insulin glulisine were identical to those of insulin human, but Fragments I and III were not (Figure 3F). Substitution of Asn^{B3} with Lys on insulin glulisine made Fragment I elute earlier than that of insulin human. Furthermore, substitution of Lys^{B29} with Glu on insulin glulisine provided an additional Glu-C endoproteinase cutting site on Fragment III, which was further digested into one polypeptide B22-B29 and a single amino acid threonine^{B30}. Peptide fragment B22-B29 eluted about 2 minutes later than Fragment III of insulin human.

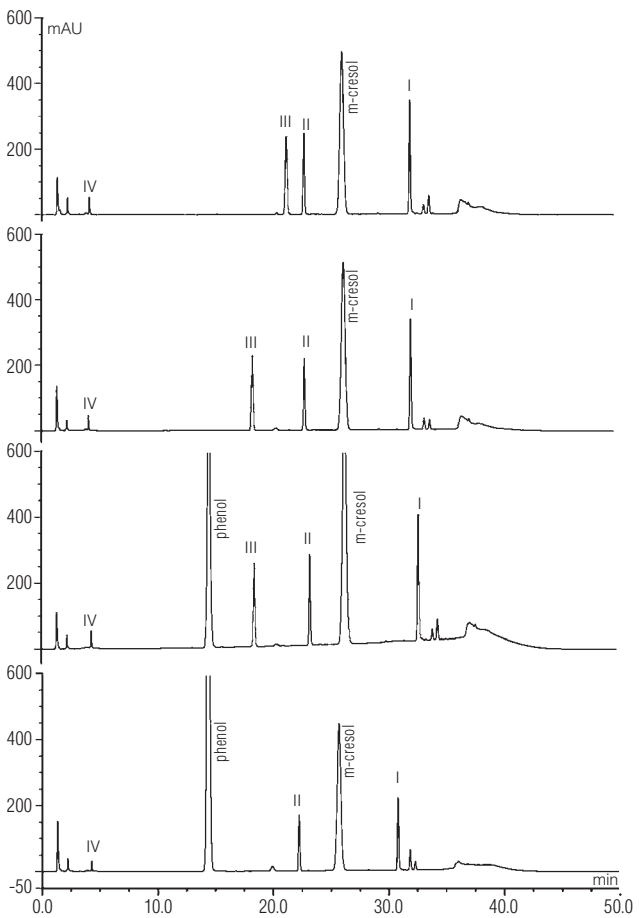

Figure 4. The insulin products (A) insulin human, (B) insulin lispro, (C) insulin aspart, and (D) insulin detemir mixed with the equal volume of their respective in-house standards separately were digested and analyzed for identification.

In order to verify that the insulin products contained the same substances as the reference standards, the digested insulin products were mixed with equal volume of the respective digested standards separately and the peptide maps of the mixtures was analyzed (Figure 4). The results confirmed the identity of the peptide fragments which presented only as single peaks without duplicates. A control group experiment was performed to verify the preservatives phenol and metacresol in the insulin products or in-house standards (data not shown). In the absence of insulin glargine and insulin glulisine products, we did not compare the peptide map between products and in-house standards.

DISCUSSION

Biopharmaceuticals are fundamentally different from conventional small molecule chemical drugs in molecular weight and complexity of the active substance, and even a subtle change in the production process can have a critical effect. The safety and efficacy profile of these biopharmaceutical products are highly dependent on the robustness and various aspects of quality. For example, under improper conditions asparagine deamidation can occur which generates aspartic acid and results in diminished bioactivity[15]. Currently, there is no method available for analyzing all the protein impurities that may be present. A validated peptide mapping method may provide a simple and accurate way to detect protein variants (Table 2)[18].

All the peptide maps of analyzed commercial products we tested were identical to their standards. Furthermore, a comparison of the maps for various insulin analogues with insulin human showed that even a single amino acid change could be detected by peptide mapping. Comparing insulin lispro with insulin human, Fragment III of insulin lispro eluted earlier than that of insulin human suggested that the

Table 2. Methods for determination of impurity and contamination[18]

Impurity	Methods
Endotoxin	Bacterial Endotoxins Test, Pyrogen Test
Host cell proteins	SDS-PAGE, Immunoassay
DNA	DNA hybridization, UV spectrophotometry
Protein mutants	Peptide mapping, HPLC, IEF, MS
Formyl methionine	Peptide mapping, HPLC, MS
Oxidized methionine	Peptide mapping, Amino acid analysis, HPLC, Edman degradation analysis, MS
Proteolytic cleavage	IEF, SDS-PAGE, HPLC, Edman degradation analysis
Aggregated proteins	SDS-PAGE, HP-SEC
Deamidation	IEF, HPLC, MS, Edman degradation analysis
Amino acid substitutions	Peptide mapping, Amino acid analysis, Edman degradation analysis

amino acid rearrangement might induce conformation change and alter the retention time of peptide fragment. Single amino acid substitution on insulin aspart changed the retention time significantly, too. This phenomenon may be caused by the increasing of peptide polarity due to the amino acid substitution from non-polar proline to polar and charged aspartic acid. Fragment III of insulin detemir was not shown on the peptide map, suggesting that the external fatty acid chain changed the polarity of Fragment III and made it invisible under our analytical condition. The reducing polarity on Fragment II of insulin glargine caused by substitution of Asn^{A21} with Gly resulted in delaying retention time. In contrast, additional 2 arginines on Fragment III increasing the polarity shortened the retention time. In terms of insulin glulisine, substitution of uncharged Asn with positive-charged Lys made Fragment I elute earlier than that of insulin human. In addition, our data showed that losing the polar amino acid threonine on Fragment III of insulin glulisine reduced polarity and extended the retention time than that of insulin human.

With the exception of the insulin detemir, all the insulin produced a single peak with a retention time of 34-35 min without protease digestion. As there was a maximum difference of only 3 amino acids between insulin human and insulin analogues, we thus concluded that the types of insulin analogue could only be successfully indentified by peptide mapping method. Peptide mapping technique displayed a capability to reveal subtle differences and to distinguish between insulin human and various types of insulin analogues more effectively than liquid chromatography did. For particular structural modifications such as those that occur in insulin detemir, the peptide mapping technique is also a useful tool for unknown peptide fragment identification. In summary, if reference standards are available, peptide mapping is a valuable initial screening tool not only for identification but also for evaluation of biopharmaceuticals. However, to further analyze impurities such as degradation products, host cell protein residues and other contaminations, it is necessary to develop other analytical tools.

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