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RESEARCH PAPER

Fingerprint Analysis of Insulin: Application in Stability Studies of Pharmaceutical Preparations

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ABSTRACT

The identification of a protein drug and the determination of its purity requires the use of several analytical methods. One identification probe used is the proteolysis of insulin with Staphylococcus aureus *protease. This method yields a characteristic pattern of peptide fragments ("fingerprint") which could be separated by reverse-phase high-performance liquid chromatography (HPLC) with gradient elution and ultraviolet detection. This probe could be used as a stability-indicating method for peptide and proteins, supplementing other traditional methods as reverse-phase (RP) and size-exclusion (SE) HPLC. Proteolysis of commercial human insulin preparations stored under different conditions produces modified fingerprints versus reference standard digested samples. Results vary as a function of the type of preparation and storage conditions since the degradation products detected by SE-HPLC are different.*

INTRODUCTION

Both proteases and restriction enzymes catalyze the hydrolysis of specific bonds in large molecules, producing an array of smaller fragments. Proteases catalyze the hydrolysis of certain amido bonds in proteins, while restriction enzymes catalyze the hydrolysis of certain phosphate bonds in nucleic acids. One way to characterize a molecule is to describe the fragments which result from a given reaction. By separating and counting the fragments, a well-known pattern emerges—"a fingerprint"—in proteins.

The analytical method most often used for separation of these fragments is the reversed-phase high-performance liquid chromatographic (RP-HPLC) technique with gradient elution and ultraviolet (UV) detection. In fact, with this technique it is possible to verify the identity of a protein or peptide and then it can be easily distinguished, for example, insulin (1–3).

Table 1 shows the protease test frequently used to verify the identity of different insulin species. For insulin, the *Staphylococcus aureus* protease V8 appeared ideal, because this enzyme cleaves peptide bonds on the carboxyl side of glutamate residues, since there are four glutamates in insulin located at strategic positions with the sequence; therefore, native insulin should be cleaved into only four fragments (see Fig. 1).

The "fingerprint" method allows the analysis of bovine, porcine, and human insulin (1–4), the identification of modified insulin (1,5), and characterization of different degradation products of the insulin during storage under different conditions (6). Darrington and Anderson (6) carried out the characterization of the covalent insulin dimer with *S. aureus* protease by comparing the digestion patterns of the dimeric insulin and native insulin in diluted acidic solutions. Since the insulin undergoes extensive deamidation and covalent amide-linked dimer formation in both solution and suspension formulations, our aim was to verify the influence of these products on the fingerprint of insulin by comparing the sample digestion patterns before and after storage at different conditions; in other words, to know its usefulness in stability studies.

MATERIALS AND METHODS

Chemicals

The *S. aureus* strain V8 used was a freeze-dried powder of 100 UI (Sigma). Human insulin was kindly given by Novo Biolabs (Spain) and pancreas bovine insulin was purchased from Sigma. Two commercial human insulin preparations were used in this study, an insulin solution and an insulin zinc suspension (see Table 2), from Novo Nordisk. Deionized water prepared with a MilliQ apparatus (Millipore Waters) was used throughout; all other chemicals and reagents were HPLC grade. All solvents were filtered with 0.45-μm (pore size) filters (Millipore).

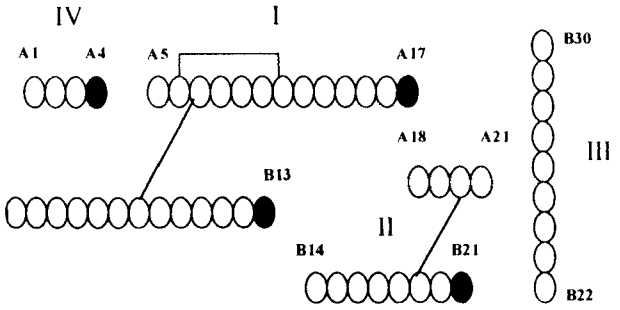


Figure 1. Theoretical *S. aureus* protease fragmentation of human insulin (●, residues of glutamine).

Analytical Methods

The instruments used were a Water Millipore apparatus consisting of a pump, model 600E Multisolvent Delivery System, an ultraviolet–visible (UV–VIS) detector, a model 490E programmable multiwavelength detector, and data adquisition software, Maxima 820. A reversed-phase C-18 column (Delta pack 300 Å, 8 · 100 mm, Waters) was used. The analysis of samples was carried out at ambient temperature and all the other chromatographic conditions were those proposed by Farid et al. (2).

Protease Digestion

Proteolysis of insulin was carried out by the method proposed by Grau (1). Digested samples were stored, without drying, at 0°C until analysis.

Table 1

The Protease Tests Most Frequently Used to Verify the Identity of Different Insulins

Insulin	Enzyme	Ref.
Human and porcine	*S. aureus*	Grau (1) Farid et al. (2) Darrington and Anderson (6)
Bovine	*S. aureus*	Farid et al. (2)
	Trypsin and *S. aureus*	Vestling (3)
	Trypsin and α-chymotrypsin	Schilling and Mitra (4)

Table 2

Composition of Commercial Human Insulin Preparations

Type of Preparation	Concentration	pH	Physical State of Insulin	Preservative	Isotonic Agents	Other Additives
Solution	40 IU/ml	7.4	Dissolution	*m*-Cresol	Glycerol	—
Insulin–zinc suspension	40 IU/ml	7.4	Crystalline	Methylparaben	NaCl	Zn^{2+}, Na acetate

Sample Treatment

In the cases of pharmaceutical preparations, 0.7-ml homogeneous samples were withdrawn from vials (≈1 mg of protein). Samples containing insulin in suspension were isolated by centrifugation, 4000 rpm for 20 min (Econospin, Sorvall Instruments Du Pont). To samples containing insulin in solution, 300 μl Zn acetate 0.0 1 M was added, and the samples were allowed to stand at 4°C overnight until precipitation of insulin and derivatives (7) was completed. Supernatants from suspensions as well as from solutions were removed and the dry residues were digested as described earlier. The samples so treated were stored at 0°C until analysis.

SE-HPLC

The method proposed by Brange et al. (8) was used. SE-HPLC was performed on a Waters Protein Pack Column (60 Å, 15 μm, 7.8 · 300 mm). The samples were appropriately diluted with the eluent.

Storage Conditions

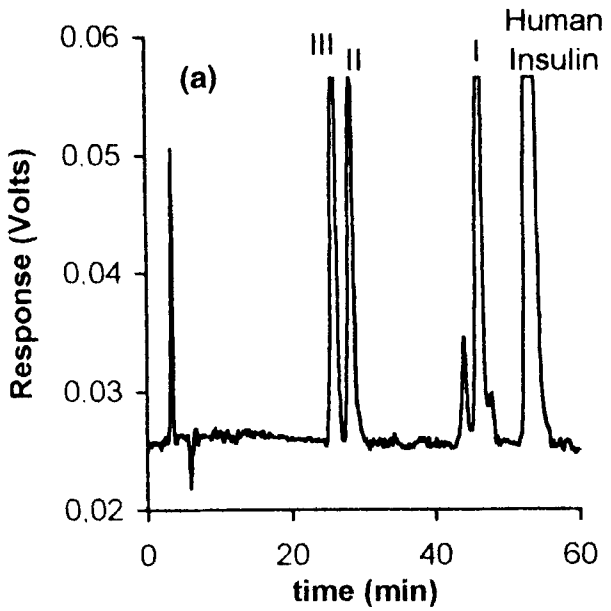
We used two commercial human insulin preparations; Table 2 shows the type of preparations and their content of auxiliary substances. The samples were stored, protected from light at different temperatures. The solution samples were stored at 50°C temperature for 70 days. In the case of suspension, samples were stored for 60 days at 50°C, as well as at room temperature (20°C) for 1 year. On the other hand, samples of both preparations were stored at 50°C with shaking at 100 rpm (Ika Vibrax, Schot Ibérica) for 7 and 10 days, respectively.

RESULTS AND DISCUSSION

Results from fingerprint analysis validation are depicted in Fig. 2. After enzymatic hydrolitic treatment, human insulin [Fig. 2(a)] shows three peaks corresponding to fragments I (elution time 46 min), II (28 min), and III (26 min) plus the peak corresponding to the unaltered insulin (54 min); two additional peaks partially fused with those of fragment I were observed, but they have not yet been identified. Bovine insulin shows a similar fingerprint analysis [Fig. 2(b)], with peaks at 46, 32, and 28 min corresponding to fragments I to III, respectively, as well as the peak corresponding to unaltered bovine insulin (50 min) and two additional unidentified peaks.

Figure 3(a) shows the chromatogram of insulin–zinc suspension after the hydrolytic treatment, showing that it is stable against the protease; similar results were obtained for the samples stored under the conditions described earlier. However, samples containing insulin in solution were hydrolyzed by the protease, and the peaks corresponding to fragments I to III, unaltered insulin and *m*-cresol, are clearly identified [Fig. 3(B)].

The results for the stored insulin dissolution samples vary as a function of storage conditions. The samples kept without shaking at 20°C were hydrolyzed by the protease and gave a fingerprint similar to that of the reference sample [see Fig. 3(B)]. The samples stored at 50°C without shaking were also hydrolyzed by protease, but the fingerprint chromatogram shows great differences compared to those obtained at the beginning. As can be seen in Fig. 4(a), peaks corresponding to fragments II and III, unaltered insulin, and the *m*-cresol are clearly identifiable, but not that corresponding to fragment I; however, additional peaks at retention times longer than that for insulin appear. These peaks are attributable to the enzymatic hydrolysis of altered insulin and could be used as a stability-indicating method. However, when the samples of insulin dissolution were stored under shaking conditions, only peaks corresponding to unaltered insulin and the conservant were observed [Fig. 4(B)]. To explain these observations, the



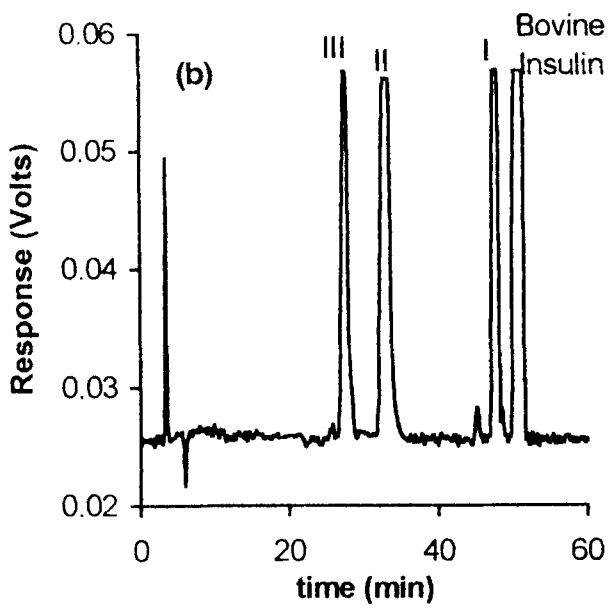
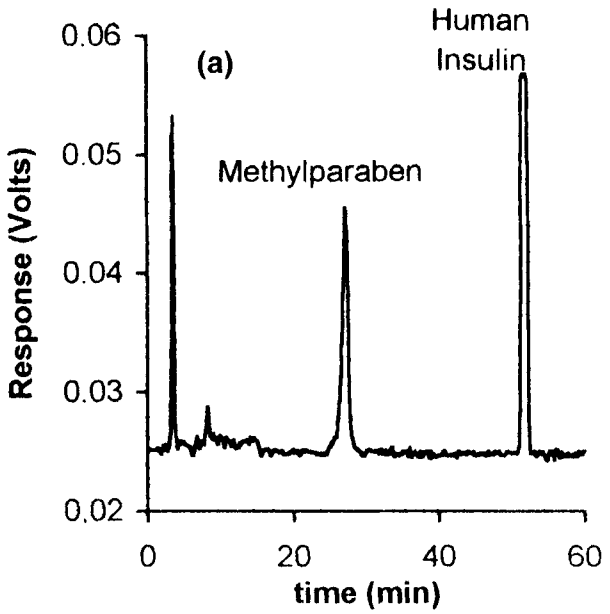


Figure 2. HPLC pattern of *S. aureus* fingerprint of human (a) and bovine (b) insulin.



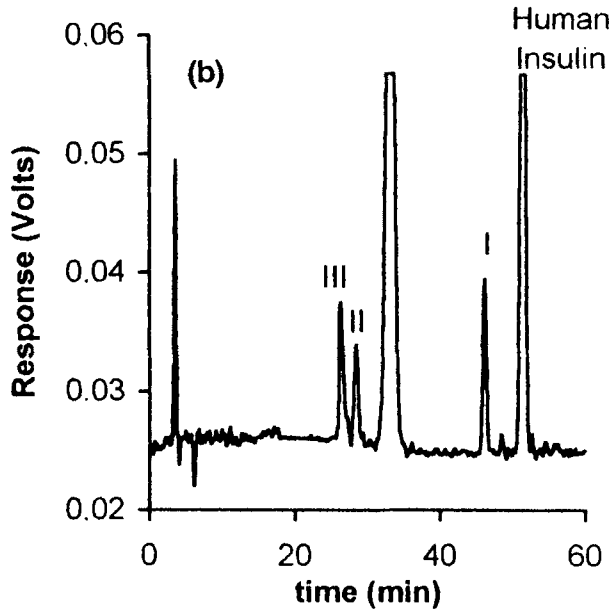


Figure 3. HPLC fingerprint of an insulin–zinc suspension (a) and insulin solution (b) samples.

samples were also analyzed by size-exclusion chromatography (SE-HPLC), and the results are depicted in Fig. 5. Chromatograms from samples stored at 20°C without shaking show the peaks corresponding to insulin and *m*-cresol [Fig. 5(A)], while those stored at 50°C show an additional peak corresponding to covalent insulin dimer [see Fig. 5(B)]. Samples stored at 50°C and under shaking conditions showed another peak corre-

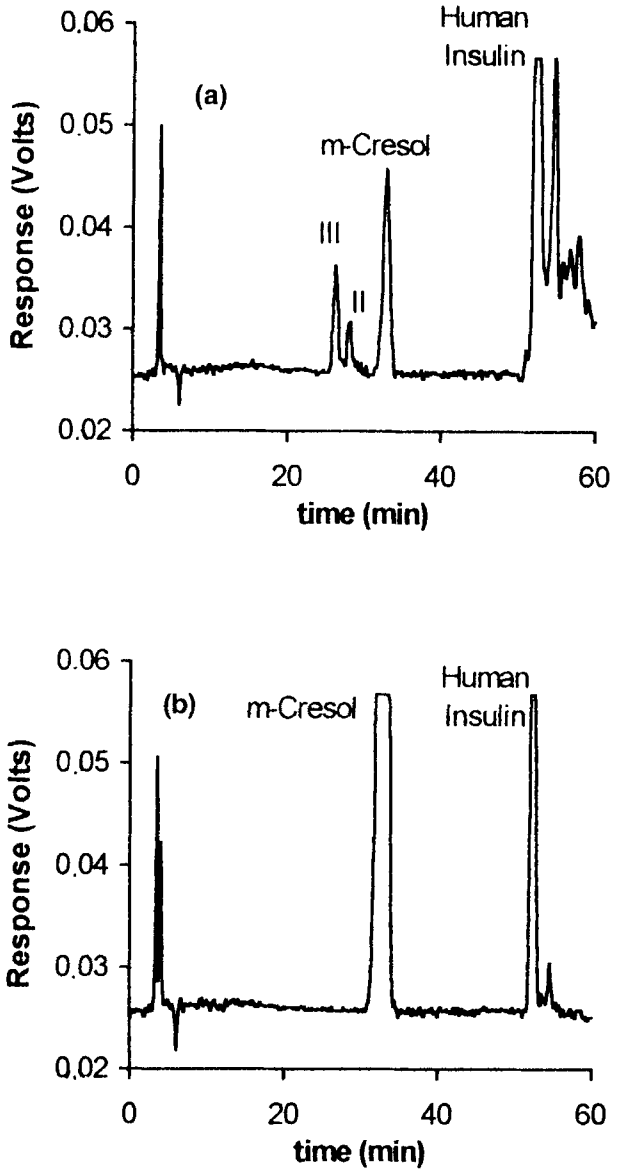


Figure 4. HPLC fingerprints of insulin solution samples stored at 50°C without shaking (a) and with shaking (b).

sponding to the higher molecular weight transformation products of insulin; this can be seen in Fig. 5(c). This is not an unexpected result because it is well known that under shaking conditions insulin forms covalent polymers; however, the resistance to the hydrolysis by the protease cannot be attributed to the full conversion of insulin to these polymers because monomer was also detected. Therefore, it cannot be concluded that the inactivation of the protease is solely due to the presence of the dimer.

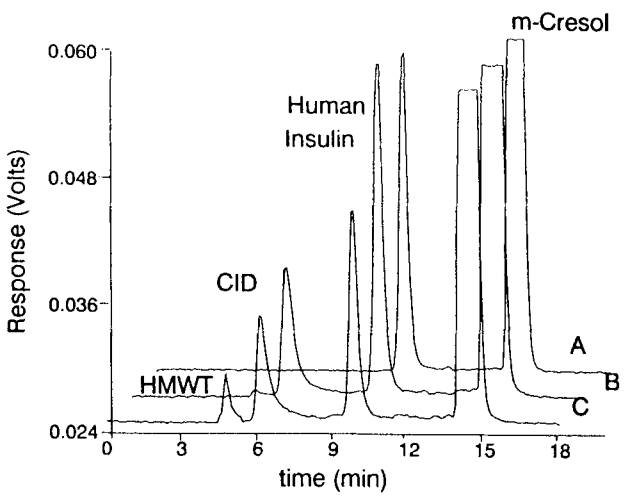


Figure 5. SE-HPLC chromatograms corresponding to solution samples stored at 20°C (a), and at 50°C without (b) and with (c) shaking (CID: covalent insulin dimer; HMWT: higher molecular weight transformation products).

The most probable mechanism able to explain the inactivity of the protease when the insulin polymer is present is adsorption of the enzyme. Further studies based on the insulin hydrolysis rate and the relative proportions of insulin dimer and protease are needed to support this explanation, but difficulties will arise from the quantification of the insulin dimer.

CONCLUSION

At present the usefulness of fingerprint analysis as a stability-indicating method is restricted to insulin solution and to cases where no covalent insulin polymers are present.

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