Chromatographic Analysis of Conformationally Changed Insulin and Its Cytotoxic Effect on PC12 Cells

Sumiko Iwasa,[a] Akina Enomoto,[a] Satomi Onoue,[a,b] Minako Nakai,[a] Takehiko Yajima,[a] and Takeshi Fukushima*,[a]

[a]*Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Toho University, 2–2–1 Miyama, Funabashi-shi, Chiba 274–8510, Japan and* [b]*Department of Pharmacokinetics and Pharmacodynamics, School of Pharmaceutical Sciences, University of Shizuoka, 52–1 Yada, Suruga-ku, Shizuoka, Shizuoka 422–8526, Japan*

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With the aim of developing a quality control system for insulin formulations in the pharmaceutical industry, we tested the feasibility of chromatographic methods, including reversed-phase high-performance liquid chromatography (RP-HPLC) and capillary electrophoresis (CE), as tools for monitoring the conformational changes in insulin. Insulin samples were dissolved in 0.01 M HCl and incubated at 60°C for 24, 48, and 96 hr to induce aggregation and fibrillation, which were confirmed by studying their circular dichroism spectra and by assaying a fluorescent indicator of β-sheet structure in aggregate proteins [thioflavine T (ThT)]; the samples were also analyzed by RP-HPLC and CE. In RP-HPLC and CE, the distinct changes in the elution patterns, as reflected by the insulin peaks, revealed the process of conformational change in insulin. With respect to the cytotoxicity of these insulin samples against rat pheochromocytoma (PC12) cells, a significant increase in lactate dehydrogenase activity was observed in the culture medium containing insulin aggregates (50 μM) as compared to the medium containing native insulin. These results suggest that the cytotoxicity of insulin in terms of the conformational changes in its structure can be rapidly assessed by using RP-HPLC and CE.

Key words —— insulin, aggregate, fibril, high-performance liquid chromatography, capillary electrophoresis, pheochromocytoma cell

*To whom correspondence should be addressed: Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Toho University, 2–2–1 Miyama, Funabashi-shi, Chiba 274–8510, Japan. Tel.: +81-47-472-1504; Fax: +81-47-472-1504; E-mail: t-fukushima@phar.toho-u.ac.jp

INTRODUCTION

The blood-glucose-regulating hormone insulin is a polypeptide composed of 51 amino acids, and it is known to undergo aggregation and fibrillation depending on the environmental conditions.[1,2] The formation of aggregates and fibrils in insulin formulations used for treating diabetes is a serious problem during the production, storage, and delivery of these pharmaceutical products. This is because misfolding of naturally occurring peptides/proteins can lead to protein aggregation and fibrillation and has thus been associated with neurodegenerative diseases[3] such as Alzheimer's and Huntington's disease. Thus far, insulin aggregation, as well as the aggregation of other therapeutic peptides/proteins, has received much attention. Winter *et al*. have reported the kinetics of insulin aggregation and its cytotoxicity against the pancreatic β-cell line RIN5fm.[4–6] A rapid and easy quality control method is required for insulin formulations.

We have previously reported the cytotoxicity of aggregated glucagon,[7] a polypeptide hormone consisting of 29 amino acids, against rat pheochromocytoma (PC12) and NIH-3T3 cells. Lactate dehydrogenase (LDH) and water-soluble tetrazolium (WST)-8 assays showed that glucagon aggregation caused cell damage and delayed cell death and that it could activate caspase-3, an apoptotic enzyme, suggesting that the cytotoxic effects of glucagon aggregates are brought about via the apoptotic signaling pathway. In our previous paper,[8] we reported that the chromatographic method of capillary electrophoresis (CE) is useful for rapidly assaying the conformational changes that lead to glucagon aggregation.

Thus, in the present study, we aimed to examine the conformational changes in insulin by using CE and reversed-phase high-performance liquid chromatography (RP-HPLC). The conformational change in insulin was induced according to the procedure described previously[4]: insulin was dissolved in 0.01 M HCl and incubated at 60°C for 1–4 days (24–96 hr). Insulin aggregation was also analyzed by traditional instrumentation techniques, including circular dichroism (CD) and techniques using thioflavine T (ThT), a fluorescent indicator for β-sheets in protein aggregates.[9]

After removing the fibrils, the insulin solution

was analyzed by CE and RP-HPLC. These techniques enable the study of conformational changes in proteins based on their distinct elution behavior.

In addition to chromatographic analysis, we evaluated the cytotoxicity induced by the conformational changes in insulin by studying the viability of PC12 cells treated with denatured insulin. On the basis of the results obtained, we discuss the relationship between elution behavior and cytotoxicity.

MATERIALS AND METHODS

Chemicals —— Insulin from bovine pancreas was purchased from Sigma Co. Ltd. (St. Louis, MO, U.S.A.); HPLC-grade acetonitrile (CH_3CN), from Kanto Kagaku Co. Ltd. (Tokyo, Japan); trifluoroacetic acid (TFA), from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan); ThT, from Wako Pure Chemicals Co. Ltd. (Osaka, Japan); Dulbecco's modified Eagle's medium (DMEM) and horse serum (HS), from Invitrogen (Carlsbad, CA, U.S.A.); fetal bovine serum (FBS), from Biowest Corp. (Nuaillé, France); and the cytotoxicity detection kit^{PLUS}, from Roche Diagnostics K.K. (Basel, Switzerland). The water used in the experiments was purified using the Milli-Q Labo system (Nihon Millipore Co. Ltd., Tokyo, Japan).

Denaturation of Insulin —— Denaturation of insulin was carried out according to the method described in a previous paper.[4] Ten mg of insulin was dissolved in 10 ml of 0.01 M HCl (1.0 mg/ml), and 4 different samples were incubated at 60°C for 0, 24, 48, and 96 hr. After incubation, the denatured insulin samples were filtered using a Millex-HV® filter (pore size, 0.45 μm; Nihon Millipore, Tokyo, Japan), and the filtrate was analyzed using a CD assay. The CE and HPLC assays were prepared as follows. An insulin solution [8.4 mg/ml in 0.01 M HCl (1.5 mM)] was prepared and incubated at 60°C for 0, 24, 48, and 96 hr. The incubated samples were diluted 8.4-fold with 0.01 M HCl (1.0 mg/ml) and then filtered using the Millex-HV filter unit. The final solution was subjected to HPLC or CE. To study the cytotoxic effects of denatured insulin on PC12 cells, we used 1.5 mM insulin in 0.01 M HCl, and the samples were diluted 10, 15, 30, and 150-fold with 0.01 M HCl (150, 100, 50, and 10 μM, respectively). These samples were incubated at 60°C for 48 and 96 hr. In these experiments, 1.5 ml polystyrene plastic tubes (Sarstedt, Germany) were used.

CD Analysis —— The incubated insulin samples were diluted 10-fold with 0.01 M HCl, and the CD spectra of aliquots of these solutions were recorded at wavelengths of 190–260 nm with a spectropolarimeter (Jasco J-720W; Jasco, Tokyo, Japan). The data acquisition interval was 0.5 nm; the scan rate, 100 nm/min. Percentages of the secondary structures of the incubated insulin were computed using a software, Jasco 32® (Jasco), with the "reed" mode as a reference.

Measurement of ThT Fluorescence —— The denatured insulin (20 μl) was sampled at the indicated time and mixed vigorously with 2.0 ml of 5.0 μM ThT prepared in 20 mM phosphate buffer (pH 6.0) and 150 mM NaCl. Subsequently, the fluorescence of the solution was measured with a spectrofluorometer (FP-6300; Jasco), with the widths of both slits were adjusted to 10 nm. The detection wavelength and excitation wavelength were set to 482 nm and 450 nm, respectively. The spectrofluorometer was placed in a temperature-controlled room (25°C), in which all the measurements were conducted.

RP-HPLC —— The Hewlett-Packard series 1050 HPLC chromatograph system (Agilent Technologies Co. Ltd., Palo Alto, CA, U.S.A.) was used in this study. The RP-HPLC column used for separation was YMC-Pack Protein-RP [150 mm × 4.6 mm; inner diameter (i.d.), 5 μm; YMC Co. Ltd., Kyoto, Japan], with a detection wavelength of 214 nm. The mobile-phase conditions used were similar to those described by Liu *et al.*,[10] but with minor modifications. We used aqueous solutions of 0.05% TFA in 20% CH_3CN (A) and 0.05% TFA in 60% CH_3CN (B) as the mobile phases; these were pumped into the column at a flow rate of 1.0 ml/min. Elution was performed with a linear gradient as follows: 1 cycle comprised 100% A pumped for 0 min, and 100% B, for 60 min. The sample (20 μl) was filtered through a Millex-HV filter (pore size, 0.45 μm; Nihon Millipore Co. Ltd.) and injected into the HPLC column with the Rheodyne 7725i injector (Rheodyne).

CE —— An HP3D CE system (Agilent Technologies Inc., Santa Clara, CA, U.S.A.) with a standard HP fused-silica capillary (i.d., 75 μm; full length, 64.5 cm; effective length, 56 cm) was used to determine the elution pattern resulting from the conformational changes in insulin. The electrolyte used was 50 mM borate buffer (pH 9.0); the voltage applied was 20 kV; and the detection wavelength was 214 nm. The sample was injected into the capillary by applying an injection pressure of 50 mbar for 2.0 s.

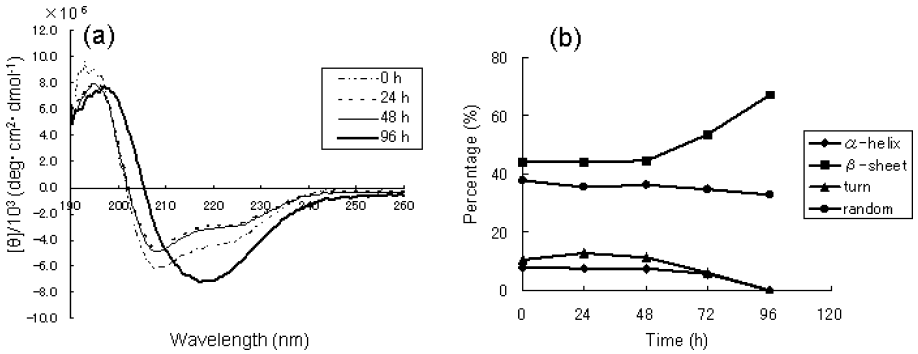

Fig. 1. CD Spectra of Insulin Subjected to Denaturation Treatment (a), and Percentage of Insulin in the Secondary Structure (b)

Cell Culture —— The PC12 cells were donated by the RIKEN BioResource Center (Ibaraki, Japan). The cells were cultured in DMEM supplemented with 5% FBS and 5% HS and incubated at 37°C in humidified air containing 5% CO_2. The cell viability assays were obtained as follows. The PC12 cells were cultivated in 96-well plates coated with poly-L-lysine (5.0×10^3 cells/well) and incubated for 24 hr; thereafter, the cells were transferred to DMEM containing 2.0 μM insulin for cell growth. Subsequently, 10 μl of the denatured insulin sample was added to each well in order to achieve the following 4 final concentrations of denatured insulin: 10, 50, 100, and 150 μM ($n = 6$). After incubating all the samples for 48 hr, the LDH assay was performed.

LDH Assay —— LDH is a marker of insulin cytotoxicity and hence the LDH present in the culture medium was quantified using a commercially available cytotoxicity detection kit^{PLUS} for LDH. The degree of cell death of the PC12 cells was measured in terms of the increase in the LDH concentration. The absorbance was measured at 490 nm using a microplate reader (Model 550; Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.).

Statistical Analysis —— The Tukey test and one-way analysis of variance (ANOVA) were used to analyze the statistical significance of the data. A p value less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Insulin Aggregation

On the basis of the methods described in previous papers,[4] we dissolved the insulin in 0.01 M HCl and heated it at 60°C to induce misfolding, aggregation, and fibrillation. The aggregation processes were studied by measuring the CD spectra and by assaying the fluorescence of ThT, a fibrillation indicator. Figure 1 (a) and (b) show the CD spectra of insulin with altered conformation had changed and the time-course changes in the secondary structure of insulin, respectively. Depending on the incubation time, the percentage of β-sheets in the insulin structure increased considerably, suggesting that insulin molecules containing a large number of β-sheets formed the fibril structures. Figure 2 shows the temporal changes in the fluorescence intensity of ThT, a fluorescent indicator of the β-sheet structure in insoluble proteins. The ThT fluorescence was observed to increase linearly with the incubation time (Fig. 2), suggesting that insulin aggregation may have occurred during incubation. These results are mostly consistent with the results of a previous report by Grudzielanek *et al.*[4]. Both the CD spectra and results of the ThT assay revealed that insulin aggregation occurred immediately under the incubation conditions used in this experiment. Thus, a structural transition from α-helix or random coil to β-sheet is the main conformational characteristic of the aggregation process of amy-

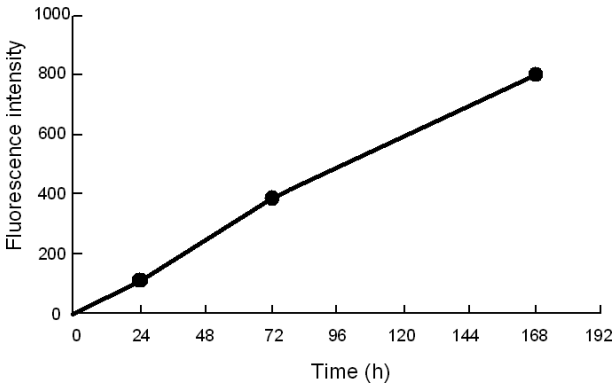

Fig. 2. Changes in ThT Fluorescence as a Function of the Incubation Time of Insulin Treated with 0.01 M HCl at 60°C

loidogenic peptides/proteins. Despite having no sequential homology, these aggregated forms share similar ultrastructural and physicochemical properties as they are both rich in β-sheets. The richness of β-sheet was also demonstrated in the aggregation of insulin.

LDH Assay

In the present study, we used PC12 cells because they have been frequently used to assess the cytotoxicity of peptide amyloids against neural cells.[7,11] In order to estimate the cytotoxicity induced by insulin aggregates and fibrils, we estimated the activity of LDH in the exudate of PC12 cells, which is considered to be an indicator of cell damage. Figure 3 shows the change in LDH activity in the culture media incubated at 48 and 96 hr as a function of the concentration of denatured insulin. As is evident from the figure, the LDH activity increased considerably in the incubated media samples containing 50, 100, and 150 μM insulin; native insulin, in contrast, did not cause any increase in LDH activity (Fig. 3). As shown in Fig. 3, samples that contained more than 50 μM insulin and that were incubated at 60°C elicited cytotoxicity. Therefore, it was confirmed that the insulin aggregates had cytotoxic effects on the PC12 cells. Moreover, Winter *et al.* reported the cytotoxicity of denatured insulin in the rat insulinoma cell line RIN-m5F[4] by using the methyl-thiazoltetrazolium (MTT)-reduction assay. All these findings reveal that when aggregation of insulin has occurred, care should be taken while using insulin formulations therapeutically.

Analysis of Denatured Insulin by Chromatographic Techniques

Thus far, the aggregation and fibrillation of denatured insulin have been well researched by means of instrumental analyses; this is in addition to studies using conventional methods such as CD, Fourier-transform IR (FT-IR), and dynamic light scattering (DLS). The morphological features of insulin fibrils have also been visualized by atomic-force microscopy (AFM). As an alternative to these expensive methods, we aimed to develop a rapid assay system using chromatographic methods, which yield an elution pattern by means of which the conformational changes and aggregation process can be clearly observed. Previously, we have used CE to demonstrate the distinct change in the elution pattern of denatured glucagon as compared to that of native glucagon. Here, we attempted to examine whether the conformational changes in denatured insulin could be detected using both CE and RP-HPLC. Since these chromatographic methods require complete dissolution of the sample in the mobile phase or electrolyte solution, we used the supernatant, namely filtrate, obtained from the denatured insulin sample, which did not contain fibrils, for the analyses. We studied the elution pattern of insulin incubated at 60°C as a function of the incubation time. Figures 4 and 5 show the representative chromatograms and electropherograms of insulin incubated for 0, 24, 48, and 96 hr, obtained by RP-HPLC and CE, respectively.

Both Figs. 4 and 5 clearly show that the peak

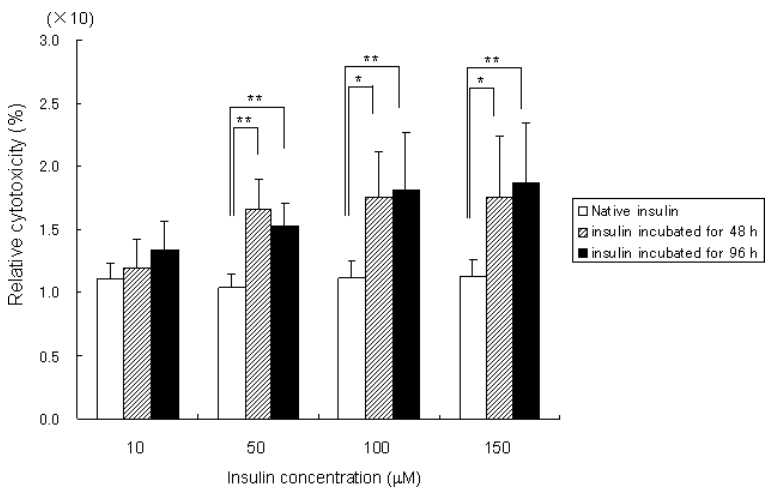

Fig. 3. Changes in LDH Activity in the Culture Medium Containing PC12 Cells after Incubation at 37°C for 48 hr as a Function of the Concentration of denatured Insulin for 48 and 96 hr

$^{*}p < 0.05$, $^{**}p < 0.01$ versus each native insulin sample.

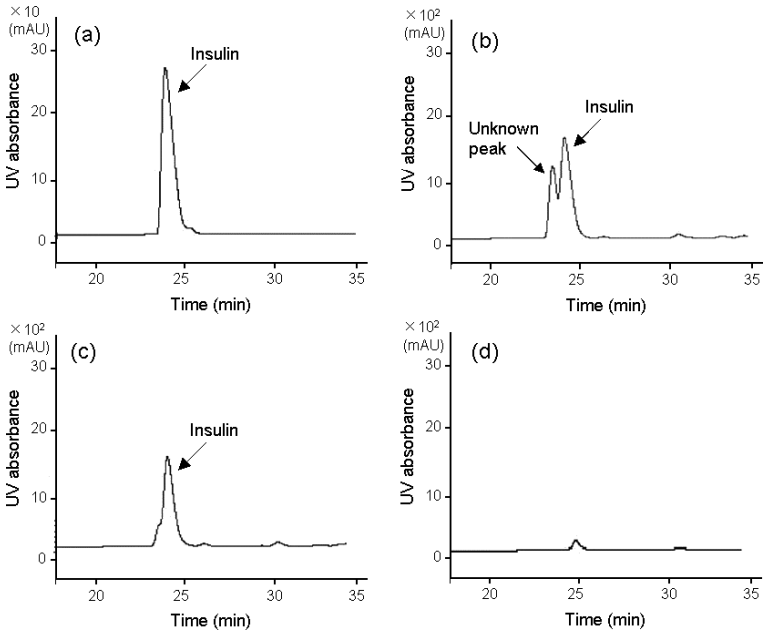

Fig. 4. RP-HPLC Chromatograms of the Denatured Insulin Samples
(a) 0 hr, (b) 24 hr, (c) 48 hr, and (d) 96 hr. Details regarding the experiment are provided in the materials and methods section.

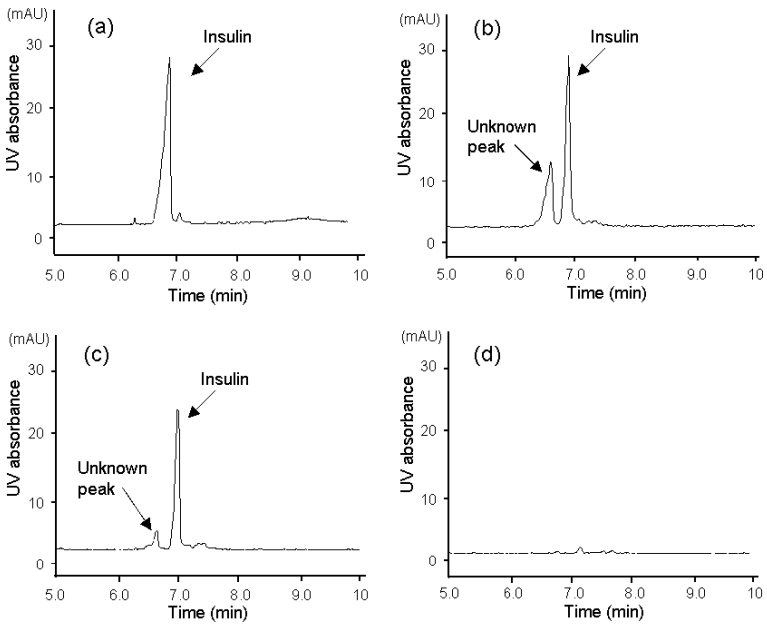

Fig. 5. CE Electropherograms of the Denatured Insulin Samples
(a) 0 hr, (b) 24 hr, (c) 48 hr, and (d) 96 hr. The electropherograms are obtained with UV detection at 214 nm, respectively. The details regarding the experiment are provided in the materials and methods section.

area of insulin reduced gradually with the incubation time, suggesting that the insulin molecules in the solution self-assembled into fibrils. At 24 hr, an unknown peak eluted at 23.7 min and a peak corresponding to native insulin (24.3 min) can be observed in the chromatogram [Fig. 4 (b)]; the fact that this new peak has an earlier retention time [Fig. 4 (b)] suggests that the hydrophilicity of denatured insulin increased. Another minor peak eluted at 30–31 min can also be observed in Fig. 4 (b). At 48 hr, the unknown peak almost disappeared in the chromatogram, and at 96 hr, the main insulin peak in the chromatogram disappeared, implying that most of the insulin molecule dissolved in the solution

formed fibrils at 96 hr.

In the electropherogram of the sample incubated for 24 hr [Fig. 5 (b)], an unknown peak (6.74 min) that eluted earlier than the peak corresponding to native insulin (6.96 min) was observed [Fig. 5 (b) and (c)]. Since the mechanism of elution of peptides or proteins in CE is complex, there is no clear explanation for the physicochemical features of the unknown peak. However, the migration time of the unknown peak reduced slightly; this suggests that the positive charge in the insulin molecule might have increased due to the conformational changes during aggregate formation. As shown in Fig. 5 (d), almost all the insulin molecules formed fibrils at 96 hr. This result is similar to the results of HPLC analysis [Fig. 4 (d)].

Although only the supernatant obtained by dissolving insulin in 0.01 M HCl and incubating it, and not the fibrils, was analyzed by RP-HPLC and CE, a distinct change in the elution pattern was observed. This result suggests that the conformation of the insulin in the supernatant could have been drastically altered during the insulin aggregation and fibrillation process. Accordingly, the insulin in the supernatant could be an indicator of the cytotoxicity of insulin aggregation and fibrillation. The relative cytotoxicity values shown in Fig. 3 suggest that denatured insulin might be harmful to living systems. A different chromatographic elution pattern of insulin formulation such as reduction of insulin peak area or appearance of newly unknown peak from that of fresh insulin formulation (Figs. 4 and 5) indicates the initiation of aggregation of insulin, which could elicit cytotoxicity.

In the present study, the structural change of incubated insulin from native insulin was not determined yet. Till now, however, it has been known that an isomerization of aspartyl and/or asparaginyl residue in some protein or peptide occurred,[12–14] and that the elution times of the isomerized peptides in HPLC analysis were different each other.[15] On the basis of these reports, we can consider that isomerization of the amino acid component might have occurred in the case of insulin under the present incubation conditions. Further studies using LC-MS/MS or chiral analysis of amino acids component in the denatured insulin are required to elucidate the structure of incubated insulin.

Chromatographic techniques can reveal conformational changes in insulin during the aggregation and fibrillation process. CE, in particular, can be used to examine the degree of denaturation of insulin in a short period of time (< 10 min) and is useful for the quality control of insulin formulations. In addition, while 0.4 ml of the sample is required for CD analysis, a small amount of the sample—of the order of nanoliters— is required for CE analysis.

In conclusion, chromatographic techniques including RP-HPLC and CE revealed that the elution behavior of denatured insulin is remarkably different from that of native insulin. Additionally, the LDH assay revealed that insulin with altered conformation is cytotoxic to PC12 cells. Therefore, the ease with which these chromatographic techniques can be carried out and the rapid manner in which they can be conducted make them useful for the quality control of insulin formulations in the pharmaceutical industry and/or in hospital laboratories.

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