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Separation of B-3 monodesamidoinsulin from human insulin by high-performance liquid chromatography under alkaline conditions

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Abstract

The separation of human insulin (HI) and related compounds such as A-21 monodesamido HI (A-21DHI) and B-3 monodesamido HI (B-3DHI) was investigated using reversed-phase HPLC and capillary zone electrophoresis (CZE). In the case of HPLC under the usual acidic conditions, whereas A-21DHI was separately eluted, B-3DHI was included in the HI peak. Then it was found that by applying a novel ODS column resistant to alkaline conditions, a good separation of B-3DHI from HI could be achieved using an alkaline eluent, and two peaks corresponding to B-3DHI were eluted in front of the HI peak.

1. Introduction

Insulin is liable to degradation by chemical reactions of its molecule with other molecules or ions in its vicinity, or to intermolecular transformations within the insulin molecule. It is well known that insulin deteriorates rapidly under acidic conditions due to deamidation at position A-21. In neutral solutions the chemical stability of insulin is improved, but during prolonged storage significant transformation of insulin still occurs, resulting from hydrolysis of Asn at position B-3 and dimerization [1]. At present, in compendia analytical methods such as the United States Pharmacopeia [2], the desamido body content of human insulin (HI) preparations is checked by reversed-phase high-performance liquid chromatography (HPLC) following the many reports on the HPLC of HI [3–5]. However, as reported earlier, the usual acidic HPLC eluents allow A-21 desamido HI (A-21DHI) to be well separated, but B-3 desamido HI (B-3DHI) is included in the main HI peak [6]. Therefore, polyacrylamide gel electrophoresis (PAGE) is often adopted in tests for some HI preparations, but the procedure for PAGE is more tedious than that for HPLC and quantitative results cannot be obtained. In this work, we tried to separate B-3DHI from the HI peak using a novel RP-HPLC column, comparing the results with those of capillary zone electrophoresis (CZE).

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2. Experimental

2.1. Materials and sample preparation

USP reference standard human insulin was used for human insulin. A-21DHI was prepared by placing HI in 0.01 *M* HCl at 40°C for 48 h and B-3DHI was produced in phosphate buffer (pH 7.4) with storage at 25°C for 1–2 weeks [1,6].

2.2. CZE analysis

CZE data were obtained using a Photal CAPI-3000 instrument (Otsuka Electronics) with a fused-silica capillary, 75 μm I.D. and 50 cm total length (37.5 cm effective length). Samples (2 mg/ml) of HI were introduced by hydrostatic injections from a height of 10 mm over 10 s. For the mobile phase, 50 m*M* sodium borate (pH 8.0)–30% methanol was used [7]. The separation conditions were 15 kV applied voltage (300 V/cm). Each component was detected by measuring the UV absorbance at 214 nm using a photodiode-array detector. The capillary temperature was maintained at 25°C.

2.3. HPLC analysis

The HPLC system consisted of a Model LC-6A pump, SPD-6A UV spectrophotometric detector, CTO-6A column oven and CR-6A Chromatopac integrator (Shimadzu, Kyoto, Japan). The absorbance was monitored at 214 nm and the column temperature was kept at 40°C. The flow-rate was 0.8 ml/min. For separation, two kinds of column, Vydac Protein & Peptide C_{18} (250 mm × 4.6 mm I.D.) from Vydac and Develosil ODS-HG-5 (150 × 4.6 mm I.D.) from Nomura Chemical, were used. For the Vydac column, the usual acidic eluent [3] was prepared as follows. For the solvent, 28.4 g of sodium sulfate were dissolved in 1000 ml of water, 2.7 ml of phosphoric acid were added and if necessary the pH was adjusted to 2.3 with ethanolamine. The eluent consisted of a mixture of solvent and acetonitrile (74:26). For the Develosil column, the mobile phase consisted of

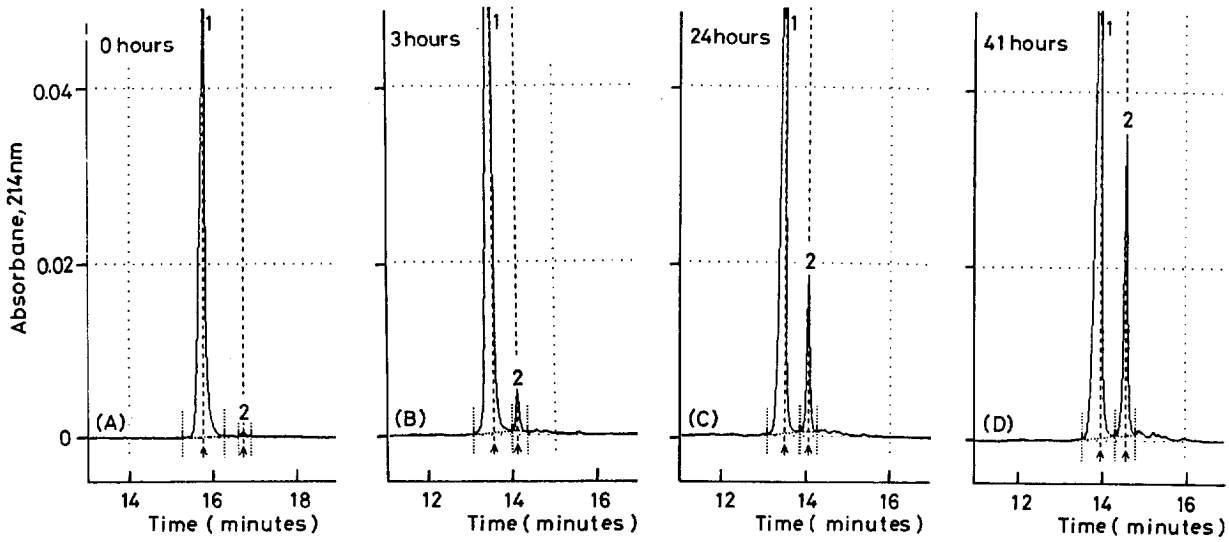

Fig. 1. CZE of HI degraded under acidic conditions for (A) 0, (B) 3, (C) 24 and (D) 41 h. Peaks: 1 = HI; 2 = A-21DHI. Capillary, fused silica, 75 μm I.D., 37.5 cm to the detector, 50 cm total length; injection, hydrostatic (10 mm, 10 s); applied voltage, 15 kV; mobile phase, 50 m*M* sodium borate (pH 8.0)–30% methanol.

0.1 *M* phosphate buffer (pH 7.4 or 9.0)–acetonitrile (76:26).

3. Results and discussion

3.1. CZE separation of HI and DHI

In line with the previous report [8], HI and A-21DHI could be well separated by CZE. Further, it was mentioned that not only A-21DHI but also B-3DHI can be determined by CZE, similarly to PAGE [9]. Samples of HI degraded under acid and/or neutral conditions were prepared and the formation of A-21DHI and/or B-3DHI was checked by CZE using our buffer conditions [7] and CZE system. As shown in Figs. 1 and 2, good resolution of A-21DHI and B-3DHI from HI was achieved. In acidic conditions, A-21DHI rapidly increased, as shown in Fig. 1. On the other hand, the peak corresponding B-3DHI increased more slowly in neutral conditions (Fig. 2). The retention times of A-21DHI and B-3DHI differ and B-3DHI eluted after A-21DHI. In Figs. 1 and 2 the retention time of each peak is different in the electropherograms because the analyses were performed at the sampling time and it was difficult to control the analytical conditions pre-

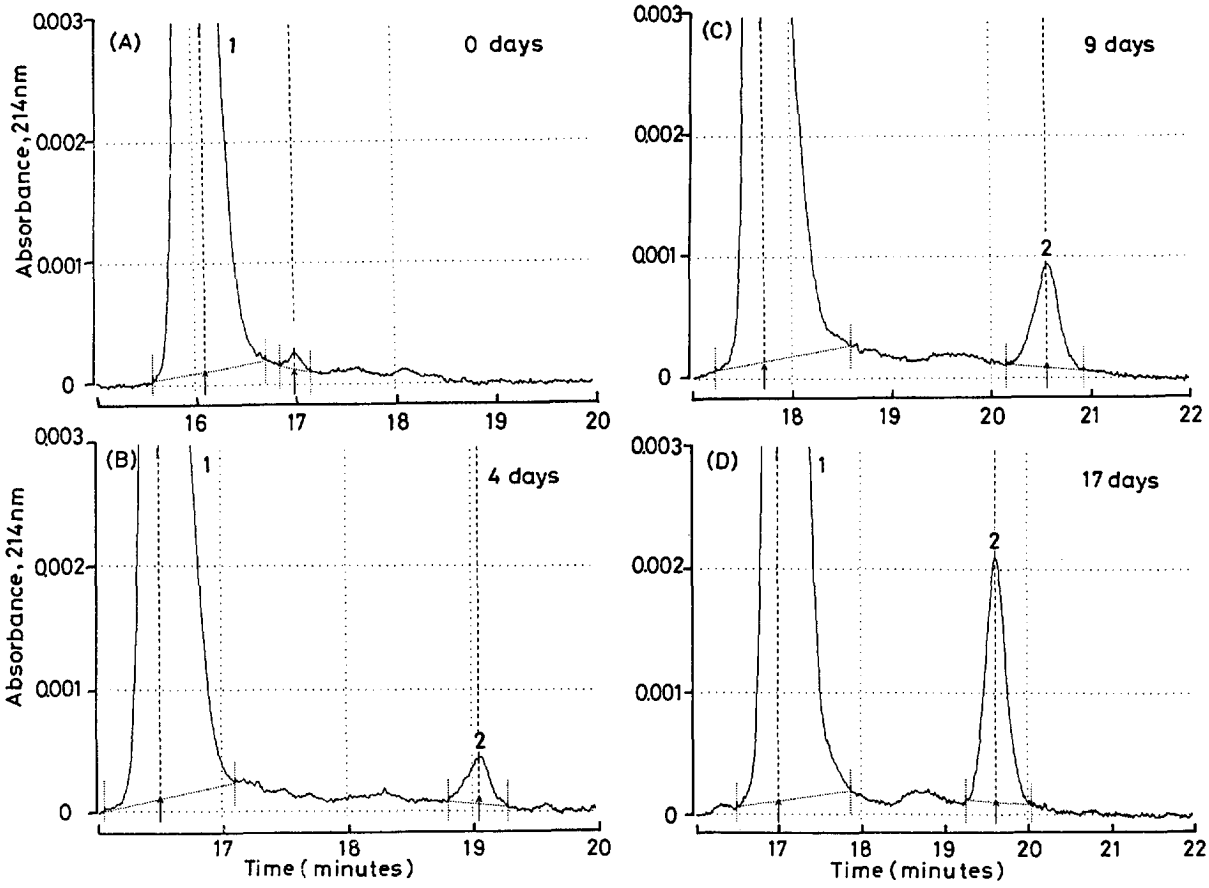

Fig. 2. CZE of HI degraded under neutral conditions for (A) 0, (B) 4, (C) 9 and (D) 17 days. Peaks: 1 = HI; 2 = B-3DHI. CZE conditions as in Fig. 1.

cisely. Using CZE, the formation of A-21DHI and B-3DHI could be followed.

3.2. HPLC separation

The separation of B-3DHI from the HI peak was tried by reversed-phase HPLC. Under the usual HPLC conditions using an acidic eluent, A-21DHI was well separated from HI, as shown in Fig. 3. However, under the same conditions, the formation of B-3DHI caused no significant chromatographic change except for the small shoulder indicated by an arrow in Fig. 4. From these results, it was considered that the desamido derivatives have an increased net negative charge due to the replacement of the side-chain amido group with an ionized carboxylate, and as a result under acidic conditions the hydrophobicity of A-21DHI is different from that of the HI molecule, but the desamidation at B-3 has no effect on the hydrophobicity of HI. Considering that B-3 Asn is deamidated only under alkaline conditions, it was suggested that B-3 Asn and/or B-3 Asp would be revealed on the surface of the HI molecule only at neutral or higher pH. Therefore, in order to try to separate B-3DHI under alkaline conditions, the recently developed pH-stable ODS column was applied. HPLC traces of B-3DHI using an alkaline eluent (pH 9.0) are shown in Fig. 5. As can be seen, two peaks eluting before the HI peak increased with B-3DHI formation. It has been reported that B-3DHI has two conformations due to rearrangement by hydrolysis, as shown in Fig. 6, and in disc gel electrophoresis two bands of B-3DHI can be detected [1,6]. It was suggested that the presence of these double HPLC peaks indicated that the two conformations of B-3DHI can be separated by RP-HPLC. Further comparison of the contents of B-3DHI and A-

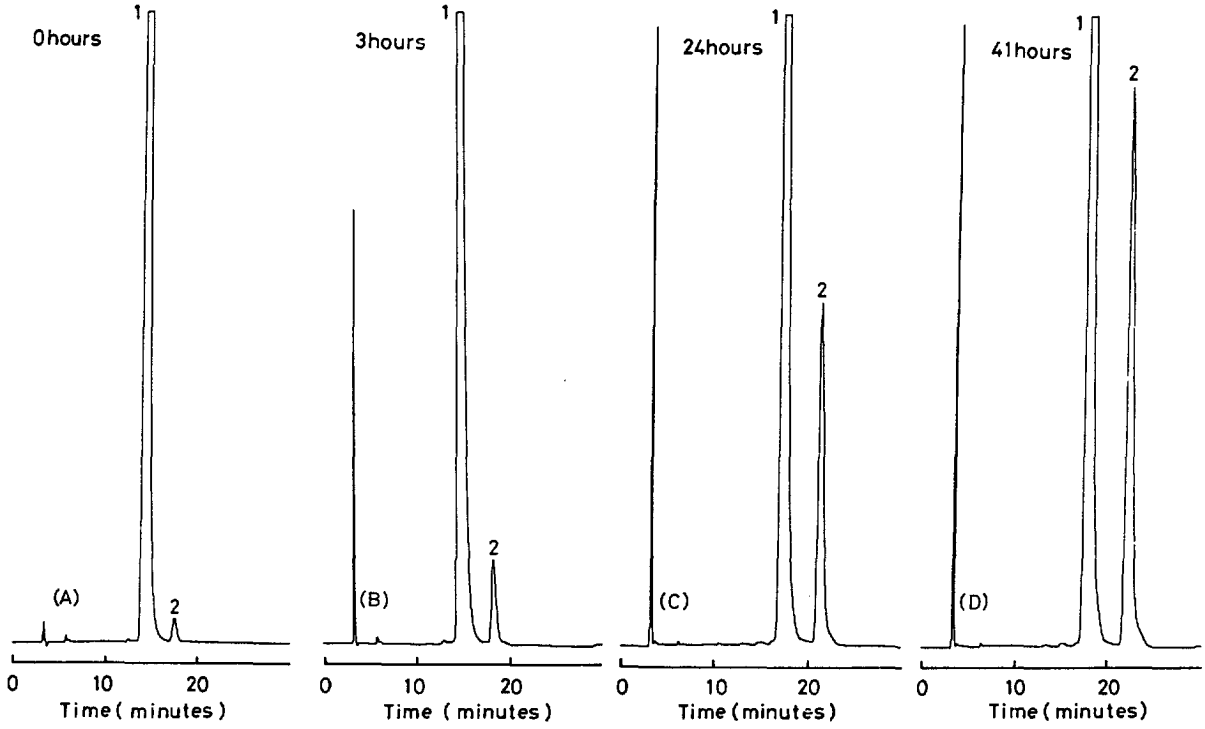

Fig. 3. HPLC of HI degraded under acidic conditions for (A) 0, (B) 3, (C) 24 and (D) 41 h. Peaks: 1 = HI; 2 = A-21DHI. Column, Vydac Protein & Peptide C_{18}; mobile phase, 0.2 *M* sodium sulfate–0.04 *M* phosphoric acid (pH 2.3) containing 26% acetonitrile.

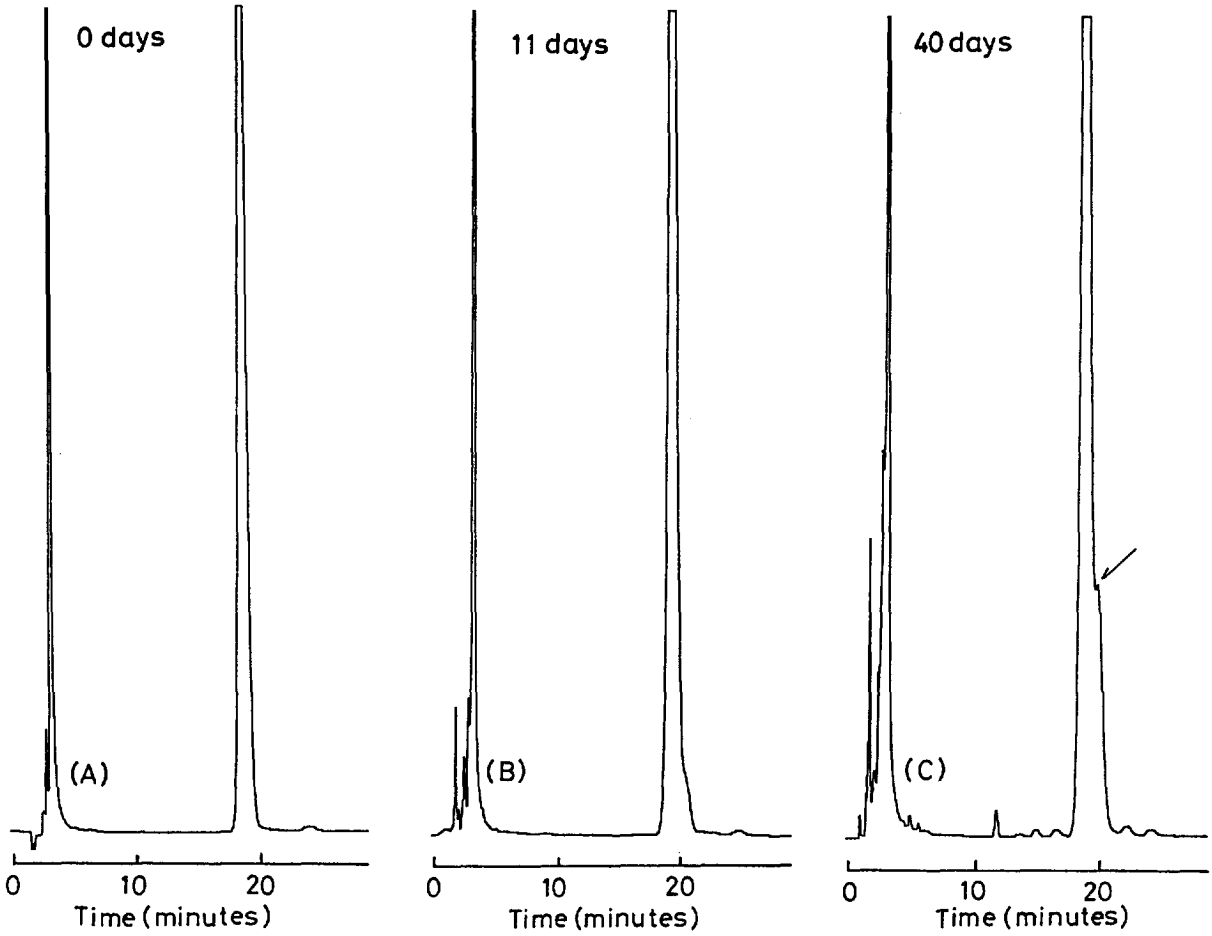

Fig. 4. HPLC of HI degraded under neutral conditions for (A) 0, (B) 11 and (C) 40 days. HPLC conditions as Fig. 3.

21DHI, determined as percentage total areas of peaks, revealed good agreement between the data from RP-HPLC and those from CZE, as shown in Table 1.

3.3. Application to the preparation of human insulin injection

Several neutral human insulin injections stored at 4°C for 1–7 years in our laboratory were analysed by CZE and RP-HPLC. To apply CZE to the preparation, pretreatment was needed in order to obtain sharp peaks, i.e., insulin was precipitated by adding zinc acetate solution and redissolved in 0.01 *M* HCl in order to remove glycerin. The CZE trace for neutral human insulin injection stored for 7 years is shown in Fig. 7. In HPLC, neutral human insulin injection was only diluted with 0.01 *M* HCl to contain 10 units/ml and injected. In the preparation of human insulin injections stored for long periods, significant amounts of B-3DHI and A-21DHI were determined, and it was found that not only B-3DHI but also A-21DHI can be better separated from HI using phosphate buffer at pH 7.4 than at pH 9.0. The HPLC trace for neutral human insulin at pH 7.4 is shown in Fig. 8.

Also in the case of insulin preparations, CZE and RP-HPLC showed similar levels of desamido formation, as shown in Table 2. From these

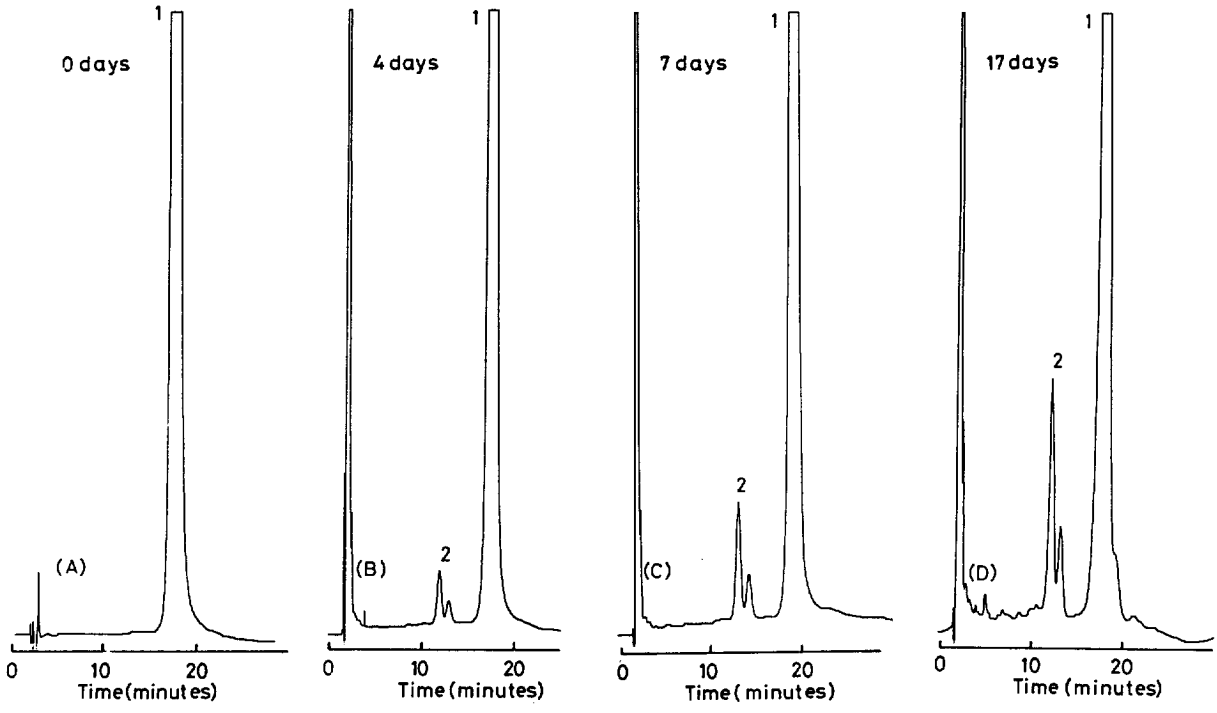

Fig. 5. HPLC of HI degraded under neutral conditions for (A) 0, (B) 4, (C) 7 and (D) 17 days. Peaks: 1 = HI; 2 = B-3DHI. Column, Deverosil ODS-HG-5 (Nomura Chemical); mobile phase, 0.1 *M* phosphate buffer (pH 9.0) containing 26% acetonitrile.

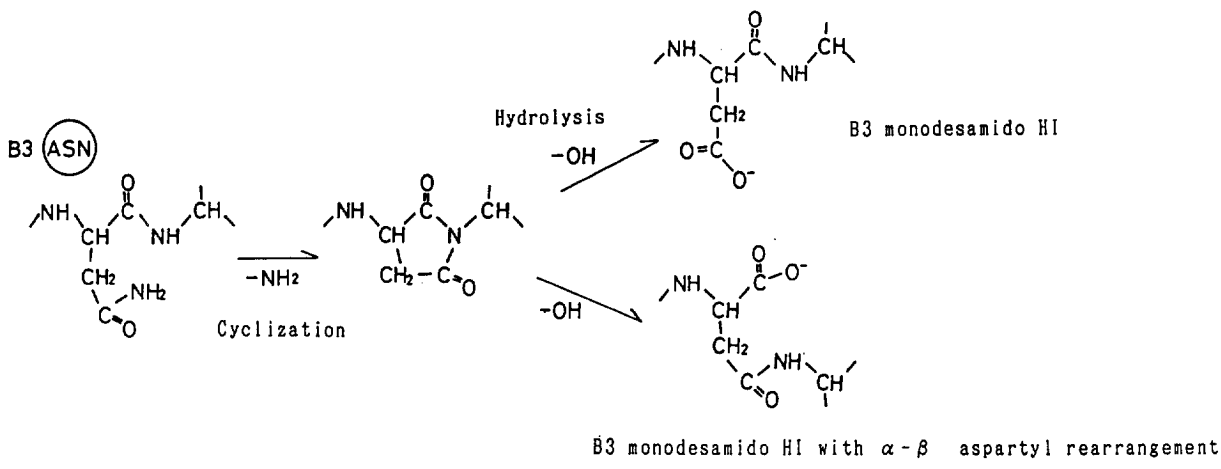

Fig. 6. Mechanism of deamidation of insulin under neutral conditions.

Table 1
Desamido formation (%) in human insulin

Time (h)	A-21DHI (%)		Time (days)	B-3DHI	
	HPLC	CE		HPLC	CE
0	0.81	0.84	0	0.28	0.23
3	2.78	2.68	4	1.64	1.43
24	15.83	13.19	10	5.78	6.35
48	27.29	24.89	20	11.32	12.08

A-21DHI was prepared in 0.01 *M* HCl at 40°C and B-3DHI was produced under neutral conditions at 40°C.

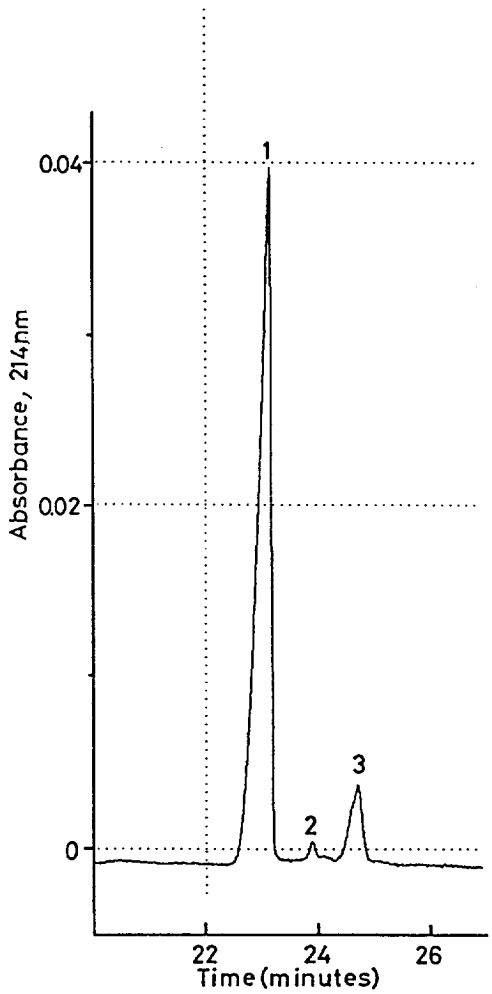

Fig. 7. CZE of neutral human insulin injection stored for 7 years. Peaks: 1 = HI; 2 = A-21DHI; 3 = B-3DHI. CZE conditions as in Fig. 1.

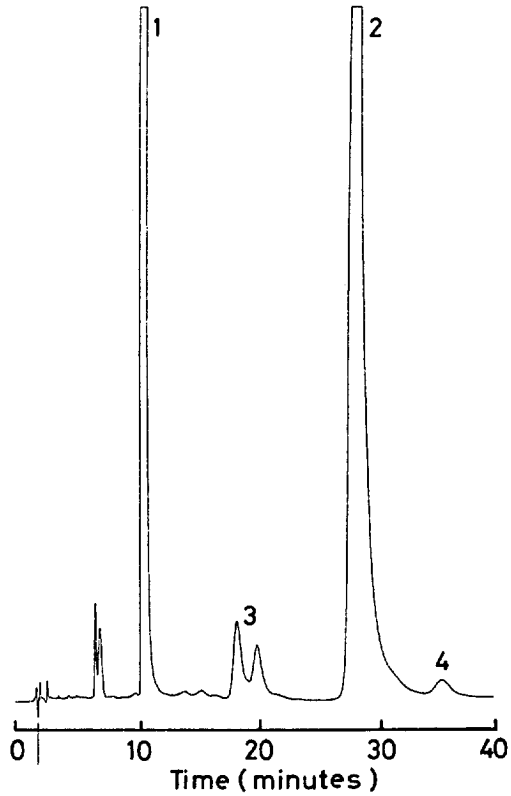

Fig. 8. HPLC of neutral human insulin injection stored for 7 years. Peaks: 1 = *m*-cresol; 2 = HI; 3 = B-3DHI; 4 = A-21DHI. Column, Deverosil ODS-HG (Nomura Chemical); mobile phase, 0.1 *M* phosphate buffer (pH 7.4) containing 26% acetonitrile.

Table 2
Desamido content (%) in neutral human insulin injection

Period stored at 4°C[a] (years)	HPLC		CZE	
	A-21DHI	B-3DHI	A-21DHI	B-3DHI
7	1.28	8.65	0.94	9.99
3	0.38	2.72	0.34	3.36
1	0.18	1.42	0.34	2.32

[a] Samples were stored in a refrigerator.

results, it was clarified that the prolonged storage of human insulin injection produced more B-3DHI than A-21DHI. Further considering that B-3DHI increases gradually when insulin is stored even at −20°C in the solid state, it is

important for quality control to analyse B-3DHI quantitatively.

4. Conclusions

RP-HPLC under alkaline conditions (pH 7.4–9) is a powerful method for detecting the formation of B-3DHI in HI powder and/or HI preparations.

Acknowledgements

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