Journal of Chromatography, 237 (1982) 511–514
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,585

Note

Separation of human insulin and some structural isomers by high-performance liquid chromatography

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(Received November 27th, 1981)

Insulin is a polypeptide hormone of molecular weight *ca.* 6000 composed of two chains (A and B) joined by two disulphide bonds. The A chain contains a further internal disulphide bond. The cystine residues involved in these bonds are apparently invariant in natural insulins[1], but the sequence of amino acids in each chain may change. At present the only insulins used therapeutically are of porcine or bovine origin. However, the synthesis of human insulin by chemical[2], semisynthetic[3] or DNA recombinant[4] methods has been described. If the A and B chains are synthesized separately and then combined, theoretically a number of disulphide bond isomers could be formed.

Reversed-phase high-performance liquid chromatography (HPLC) has proved to be a powerful technique for the analysis of insulins, capable of separating human, bovine and porcine insulins[5,6] although human and porcine insulin differ in only a single amino acid residue in the B chain and bovine and porcine insulin differ in only two amino acid residues in the A chain.

We have now examined the behaviour of synthetic human insulin, a disulphide bond isomer and several D-Cys analogues[7,8] in two reversed-phase HPLC systems[6,9] and suggest that such systems may be suitable for detection of by-products in the synthesis of insulin.

EXPERIMENTAL

Materials

Chemicals and solvents were obtained from BDH (Poole, Great Britain) and were of "AnalaR" grade where possible. Other materials were: L(+)-tartaric acid from Sigma (Poole, Great Britain), acetonitrile (HPLC grade) from Fisons (Loughborough, Great Britain) and 2-methoxyethanol (autoanalyser grade) from Koch Light (Colnbrook, Great Britain).

Synthetic human insulin, three analogues synthesized with D-cysteine at positions A7, A11, and B7, respectively, and the disulphide bond isomer A7-A11, A6-B7 were obtained from Ciba-Geigy (Basle, Switzerland). Because of their limited availability all except the first of these insulins had been recovered by Ciba-Geigy from previous spectroscopic experiments. Each sample was dissolved in 0.01 *M* hydrochloric acid to give a nominal concentration of 2 mg/ml.

0021-9673/82/0000–0000/$02.75

HPLC

An Altex Model 110A pump and a Cecil CE 2012 variable-wavelength UV monitor were used. Injections were made with a Rheodyne 7125 sample-injection valve fitted with a 20-μl loop. A precolumn (50 × 5.0 mm I.D.) dry packed with LiChroprep Si 60 (15–25 μm, Merck, Darmstadt, G.F.R.) was fitted to the system before the injection valve. Separations were performed at ambient temperature on either a prepacked 250 × 4.6 mm I.D. column of Zorbax-TMS (6 μm) (Dupont, Hitchin, Great Britain) or a 150 × 5.0 mm I.D. column of ODS Hypersil (5 μm, Shandon Southern, Runcorn, Great Britain) slurry packed in propan-2-ol. Solvent mixtures were degassed in an ultrasonic bath and filtered before use. Two systems were examined:

System I (Zorbax TMS column). The mobile phase was prepared as follows: solution A: 0.1 *M* Sodium dihydrogen orthophosphate in water–2-methoxyethanol (95:5) adjusted to pH 2.0 with orthophosphoric acid; solution B: acetonitrile–2-methoxyethanol (95:5)[10]. These solutions were mixed in the proportions 74% A–26% B. The column eluate was monitored at 210 nm.

System II (ODS Hypersil column). The mobile phase consisted of acetonitrile–0.1 *M* ammonium sulphate (25:75) + 0.005 *M* L(+)-tartaric acid in water pH 3.0. Cetrimide was added to give a final concentration of $14 \cdot 10^{-6}$ *M*. The eluate was monitored at 225 nm.

Thin-layer chromatography (TLC)

TLC was performed on plates precoated with microcrystalline cellulose (Schleicher and Schüll G1440) in two systems[2]: *System 155:* pentan-1-ol–pyridine–water–butan-2-one–formic acid (40:28:15:11:5) and *system 112E:* butan-1-ol–pyridine–water–formic acid (44:24:20:2). The spots were located with ninhydrin.

RESULTS AND DISCUSSION

Separations of human insulin and its analogues in the two HPLC systems are shown in Fig. 1, and the capacity factors (k') obtained from each compound are listed in Table I. The k' quoted is for the major peak in each sample as some heterogeneity (not detected by TLC) was observed. All analogues were well separated from human insulin in both HPLC systems and almost complete resolution was achieved using system I. The order of elution was similar in both cases although the disulphide bond isomer eluted before the D-Cys B7 analogue in system I (Fig. 1A) but after, and only partially resolved from it, in system II (Fig. 1B). The D-Cys A11 analogue showed the greatest difference in retention behaviour relative to human insulin. This was most marked in system II.

The TLC results are presented in Table I. The chromatographic mobilities of the analogues relative to those of insulin are in good agreement with published results[7]. However the discrimination of the TLC systems is not as great as that of the HPLC systems. Although TLC possesses advantages as a rapid screening procedure, analyses by HPLC provide more detailed information.

The behaviour of these synthetic insulins on HPLC confirms previous work showing that the analogues appear to possess more hydrophobic character than native insulin[11]. For the diastereoisomers, the effect of substitution on the retention

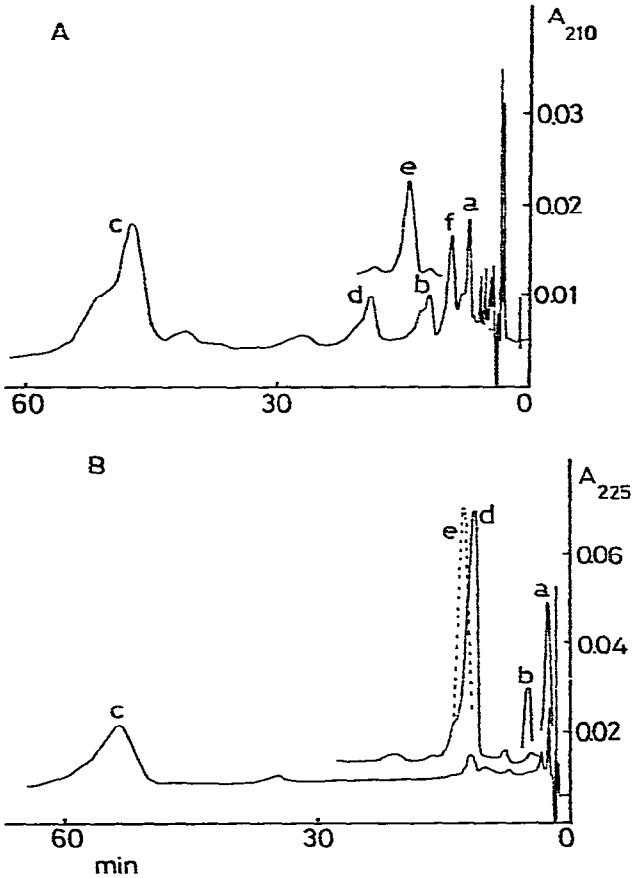

Fig. 1. Composite chromatograms showing the behaviour of synthetic human insulin and some analogues. A, Column: Zorbax TMS; solvent: see text; monitored by UV at 210 nm. B, Column: ODS-Hypersil; Solvent: see text; Monitored by UV at 225 nm; Peaks a and b 1/10 actual size. Analyses conducted at ambient temperature, flow-rate 1.0 ml/min. a = Synthetic human insulin; b = analogue with D-Cys A7; c = analogue with D-Cys A11; d = analogue with D-Cys B7; e = disulphide isomer A7-A11, A6-B7.

time reflects the extent of conformational change necessary to accommodate the appropriate D-Cys residue as predicted from examination of the crystal structure of native insulin[12]. All three substitutions appear to increase the accessibility of residues in the hydrophobic "core" of the molecule, thus explaining the observed increase in hydrophobic behaviour. On the same basis[12], the interchange of the disulphide bonds at residues A6 and A7 might also be expected to produce a considerable conformational change in the molecule with comparable effects. The chromatographic behaviour of the disulphide bond isomer suggests that this is indeed the case.

Insulin readily degrades to monodesamidoinsulin in solution, especially at acid pH, by deamidation of the terminal asparagine residue at A21. Analysis of mixtures of porcine, bovine, and their respective monodesamidoinsulins is not practicable under simple isocratic conditions because native porcine insulin and beef monodesamidoinsulin coelute. However, such mixtures may be completely resolved by gradient elution[13] or by use of a hydrophobic ion-pair system[9]. The latter, employed as system

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF SYNTHETIC HUMAN INSULIN AND SOME ANALOGUES

For details of chromatographic systems see text.

	HPLC k'		*TLC**	
	System I	*System II*	*System 155*	*System 112E*
Human insulin	1.9	1.2	1.0 (R_F 0.08)	1.0 (R_F 0.31)
D-Cys A7	4.1	3.1	1.52	1.14
D-Cys A11	21.9	43.7	2.10	1.23
D-Cys B7	7.2	8.9	1.48	1.12
Disulphide bond isomer A-7-A11, A6-B7	5.1	9.7	1.90	1.15

* Migration distances on microcrystalline cellulose relative to human insulin.

II here, is also able to separate the isomeric analogues of human insulin that we have studied. It will not, however, resolve human and porcine insulins.

The availability of insulin analogues of defined structure has enabled us to assess the value of HPLC for their separation. Our results demonstrate the potential of such a technique for analysis of insulin obtained by synthesis, in particular in separating disulphide bond isomers. The differences in behaviour of the analogues in the two HPLC systems employed, however, and the inability of one system to separate human and porcine insulins having different amino acid sequences, suggest that undue reliance should not be placed on homogeneity of such complex molecules in a single HPLC system.

ACKNOWLEDGEMENTS

We are most grateful to Dr. W. Rittel, Ciba-Geigy Ltd., Basle, for supplying us with the human insulin and analogues. The TLC experiments were carried out by Miss J. Davidson.

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