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IDENTIFICATION AND ISOLATION OF HUMAN INSULIN A AND B CHAINS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the isolation, identification and quantification of human insulin A and B chains by high-performance liquid chromatography (HPLC) is described. These chains were isolated from a peptide mixture produced by *E. coli* with modified genes obtained by genetic engineering. The method is based on the use of hydrophilic reagents, forming ion pairs in a reversed-phase column. Because some undesirable effects resulting from the use of phosphoric acid were observed, especially with the B chain, a new HPLC method was developed for each of the two human insulin chains. The use of trifluoroacetic acid as a counter ion for the A chain and of formic acid for the B chain led to the rapid isolation and purification of each chain by HPLC. The advantage of this method is that it provides a highly pure product, which was identified by polyacrylamide gel electrophoresis and amino acid analysis.

INTRODUCTION

To avoid the problems encountered by patients receiving porcine or bovine insulin therapy to control diabetes[1], an interesting alternative is to administer an analogue of insulin, the structure and function of which are similar to that of human insulin. Therefore, the genes for both the A and B chains of human insulin were synthesized *in vitro*[2] and were inserted separately into two distinct strains of *E. coli*[2] in order to achieve the production of hybrid polypeptides, containing β-galactosidase, coupled to either the A or the B chain. On recovery and cleavage with cyanogen bromide[3] of the hybrid polypeptides[4], a mixture of peptides was obtained, including

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the A or B insulin chains. As this peptide mixture is insoluble in phosphoric acid[5] and as, in our hands, the use of phosphoric acid produced several undersirable effects, especially during the recovery of the B chain for lyophilization, we sought a more suitable solvent. Of the hydrophilic reagents that form ion pairs, which are used in the separation of peptides[6], formic acid was selected for the isolation of the insulin B chain because the peptide mixtures were completely soluble in it and because the B chain standard, dissolved in formic acid, also gave very good resolution. Trifluoroacetic acid (TFA) was selected for the insulin A chain. In both instances, the respective mobile phases selected gave sample k' values in the correct range[7]. Lyophilization of the products yielded white solids that easily dissolved in their respective solvents, thereby permitting characterization of the products by polyacrylamide gel electrophoresis and by amino acid analysis. The products so obtained were in a form that could undergo chain recombination[4,8].

EXPERIMENTAL

Apparatus

Two different chromatographic systems, both from Spectra-Physics (Santa Clara, CA, U.S.A., and Darmstadt, F.R.G.) were used. The first was an SP8750 modular analytical system with a Rheodyne (Berkeley, CA, U.S.A.) injector loop (10 μl), an SP8700 solvent delivery system, an SP8400 variable-wavelength detector and an SP4100 computing integrator. The second was an SP8000 semi-preparative system with a 100-μl injector loop. In both systems a Waters Radial-Pak μBondapak C_{18} cartridge (10 cm × 8 mm I.D. 10 μm), contained in a Z-Module Radial Compression Separation System (Waters Assoc., Milford, MA, U.S.A.), was used. Samples and solvents were clarified by filtration through porous membranes (0.22 μm) from Millipore (Milford, MA, U.S.A.).

Reagents

HPLC-grade water was obtained from triply distilled water by using a Norganic Filter Apparatus (Millipore XX1500-710). Acetonitrile (LiChrosolv grade) was obtained from Merck (Darmstadt, F.R.G.), and trifluoroacetic acid (Sequanal grade) from Pierce (Rockford, IL, U.S.A.). Formic acid (J. T. Baker, Phillipsburgh, NJ, U.S.A.) was redistilled under vacuum (20 mmHg). The A and B insulin chains (Sigma, St. Louis, MO, U.S.A.) used as standards were from porcine pancreas (I-3505) and from bovine pancreas (I-5500). Acrylamide was obtrained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Procedure

Both eluent A [0.0125% TFA (pH 3.2) in ultra-pure water for A chain and 0.5% formic acid (pH 2.7) in ultra-pure water for B chain] and eluent B (acetonitrile) were filtered through a porous membrane (0.22 μm) and degassed with helium. The A or B chains used as standards, dissolved in eluent A, were filtered through a porous membrane (0.22 μm) and were chromatographed individually to determine their retention times (Figs. 1 and 2). The external standard procedure was used to quantify the products. Various amounts of the A chain (2.7–18.3 μg) and B chain (5–40 μg) were used individually to determine the linearity of the response. To calculate the

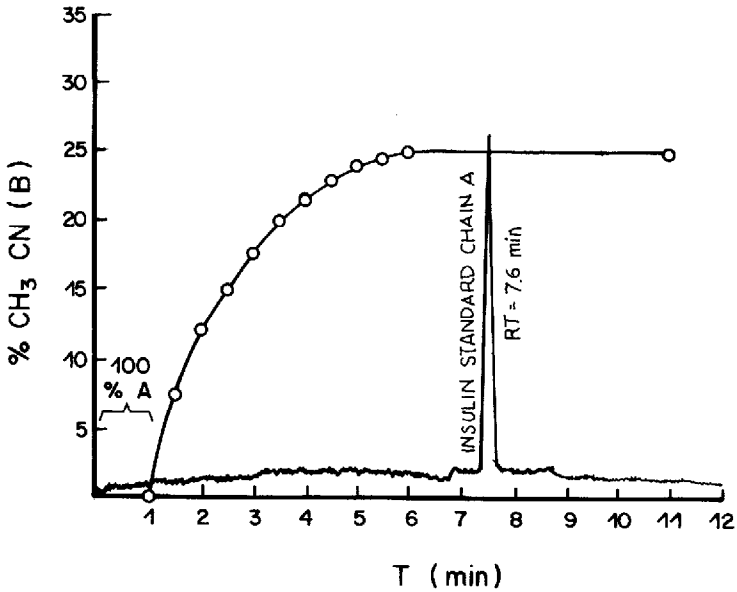

Fig. 1. Schematic chromatogram, showing retention time of A chain. Spectra-Physics system: column, Radial-Pak μBondapak C_{18} (10 cm × 8 mm I.D., 10 μm); mobile phase, 100% eluent A (1 min), followed by a convex gradient from 0 to 25% acetonitrile (eluent B) in 0.0125% TFA (pH 3.2) (eluent A); flow-rate, 2.5 ml/min; UV detection (280 nm).

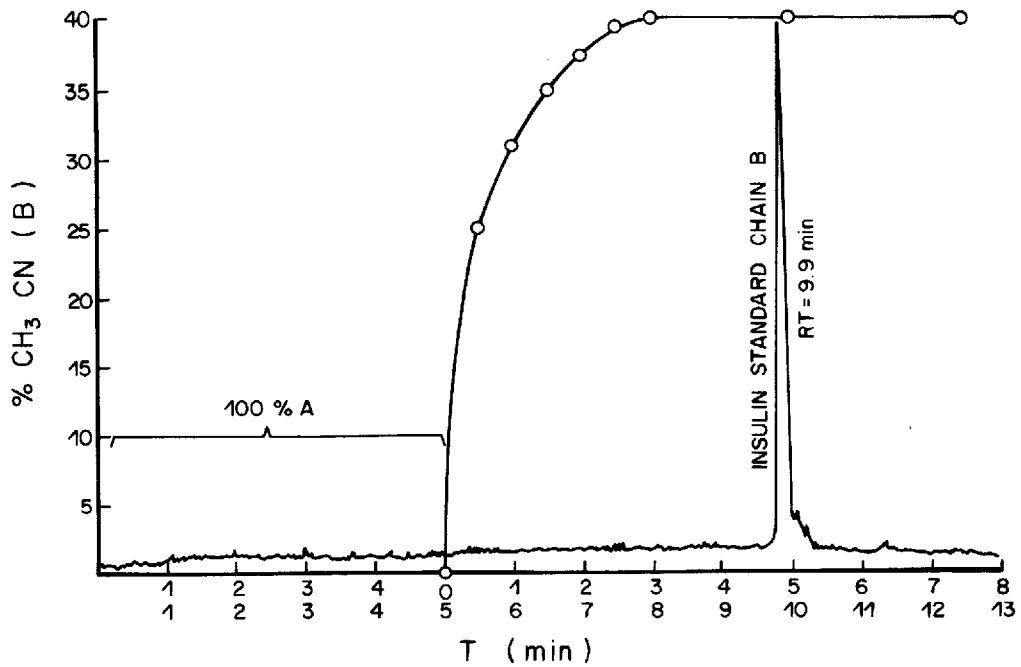

Fig. 2. Schematic chromatogram, showing retention time of B chain. Spectra-Physics system: column, Radial-Pak μBondapak C_{18} (10 cm × 8 mm I.D., 10 μm); mobile phase, 100% eluent A (5 min), followed by a convex gradient from 0 to 40% acetonitrile (eluent B) in 0.5% formic acid (pH 2.7) (eluent A); flow-rate, 2.5 ml/min; UV detection (280 nm).

TABLE I

STATISTICAL EVALUATION OF RESULTS FROM HPLC OF INSULIN A AND B CHAIN STANDARDS

Both standards were run five times at each concentration with the same chromatographic conditions, as in Figs. 1 and 2.

Chain	*Sample (μg)*	*Standard deviation (μg)*	*Confidence limit (μg)*
A	2.5– 5	0.03994	± 0.0351
	10.0–15	0.259	± 0.0227
	15.0–20	0.08064	± 0.07924
B	5.0–10	0.1179	± 0.189
	10.0–15	0.08809	± 0.09998
	20.0–40	0.160076	± 0.18167

confidence limits of the method[9], both standards were run five times at each concentration (Table I).

Chromatographic conditions

The Spectra-Physics system was programmed to provide a stepwise convex gradient elution to separate the A or B chains of human insulin from the mixture of peptides. To increase the differences between the k' values of the peaks of the peptide

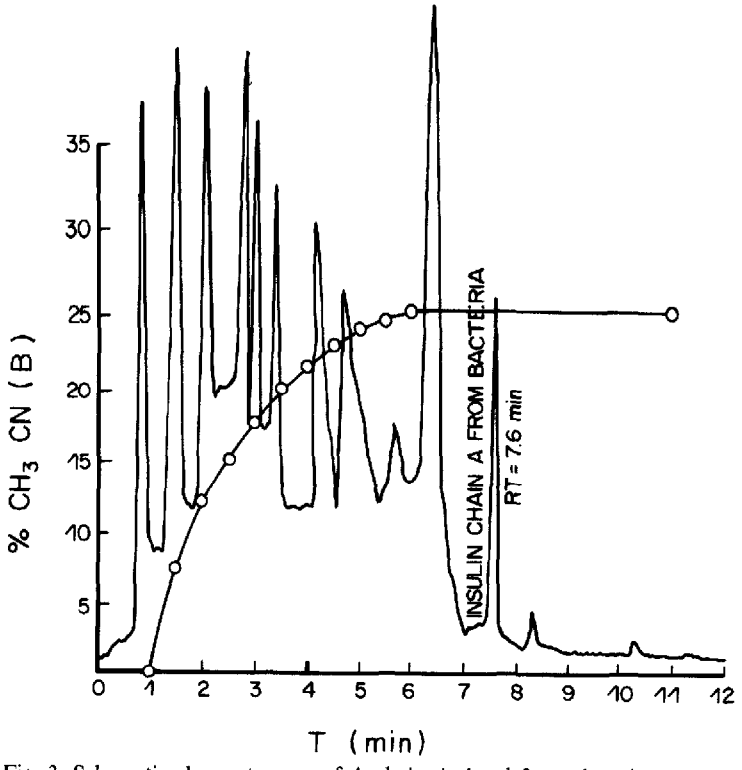

Fig. 3. Schematic chromatogram of A chain, isolated from the mixture of peptides. Chromatographic conditions as in Fig. 1.

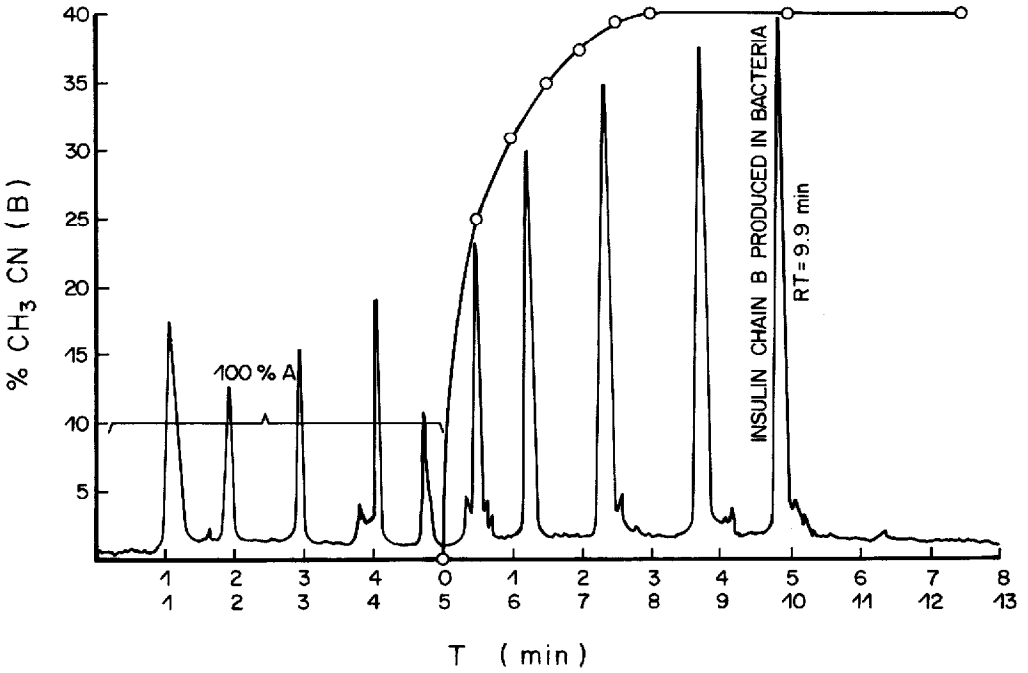

Fig. 4. Schematic chromatogram of B chain, isolated from the mixture of peptides. Chromatographic conditions as in Fig. 2.

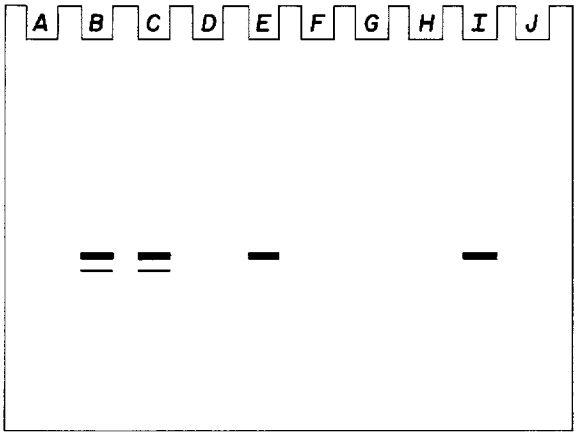

Fig. 5. Schematic electropherogram: polyacrylamide gel electrophoresis of insulin A chain produced in bacteria and purified by HPLC was carried out in a continuous gel, 16% acrylamide, Tris–H_3BO_3. Lanes B and C: 3 and 5 μg, respectively, of insulin A chain, purified by HPLC, showing one major band that migrates with the standard. lanes E and I: 5 μg of standard insulin A chain. Lanes A, D, F, G, H and J: without samples.

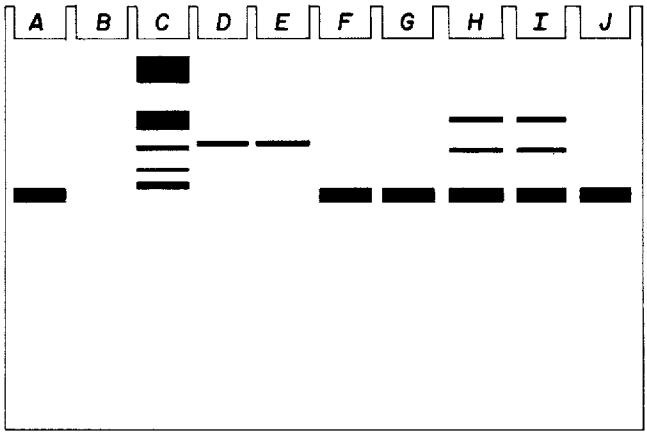

Fig. 6. Schematic electropherogram: polyacrylamide gel electrophoresis of insulin B chain produced in bacteria and purified by HPLC was carried out in a continuous gel: stacking gel, 18% acrylamide, Tris–HCl, 2.12 *M*, pH 9.18. Separation gel, 18% acrylamide, Tris–H_2SO_4, 0.0267 *M*, pH 6.1. Samples were dissolved in 7 *M* urea, Tris–H_3BO_3, 0.205 *M*, pH 8.6, diluted with water (1:5). Lanes A, G and J: 6 μg of bovine chain B used as standard and chromatographed by HPLC. Lane C: unknown peptide mixture separated from chain B by HPLC. Lane D and E: other peptides separated from chain B by HPLC. Lane F: *ca.* 7 μg of B chain, purified by HPLC, showing a single band that migrates with the standard. Lane H and I: 5 and 10 μg, respectively, of the peptide mixture before HPLC.

TABLE II

AMINO ACID ANALYSIS OF INSULIN A CHAIN, PRODUCED IN BACTERIA, AND OF THE A CHAIN STANDARD

Approximately 20 μg of porcine A chain and 20 μg of *E. coli* A chain purified twice by HPLC were hydrolysed and analysed in parallel.

Amino acid	*A chain from bacteria*		*A chain used as standard*	
	Found	*Predicted*	*Found*	*Predicted*
Aspartic acid	1.85	2.0	1.75	2.0
Threonine	0.93	1.0	1.11	1.0
Serine	2.14	2.0	2.09	2.0
Glutamic acid	3.83	4.0	3.45	4.0
Glycine	1.12	1.0	1.31	1.0
Valine	0.38	1.0	0.49	1.0
Isoleucine	1.43	2.0	1.12	2.0
Leucine	1.96	2.0	1.47	2.0
Tyrosine	2.02	2.0	1.30	2.0
Cysteine	N.D.*	4.0	N.D.*	4.0

* N.D. = not determined.

mixture and the peaks of human insulin A or B chain, pure eluent A was used for a certain period of time: for the A chain 1 min (Fig. 1) and for the B chain 5 min (Fig. 2); after this time, a convex-shaped gradient of a mixture of eluent A and eluent B was started. For the A chain the limiting buffer was TFA (pH 3.2) in 25% acetonitrile (see legend to Fig. 1), and for the B chain formic acid (pH 2.7) in 40% acetonitrile (see legend to Fig. 2). The mobile phase was pumped at a flow-rate of 2.5 ml/min and the column effluent was monitored at 280 nm.

Chromatography was performed at room temperature using a μBondapak C_{18} cartridge. The injection volume of the sample was 10 μl for the analytical determinations and 100 μl for the isolation and purification of human insulin A and B chains.

RESULTS AND CONCLUSIONS

That both the A and B chains were separated from the peptide mixture (Figs. 3 and 4) by use of this HPLC method is demonstrated by the well resolved peaks corresponding to the standards. The recovered materials were re-chromatographed individually in the same systems and yielded peaks containing the purified A and B chains, respectively.

The very high purity of the products was demonstrated by polyacrylamide gel electrophoresis of the samples, in which a major band, migrating with the appropriate standard, was observed in each instance (Figs. 5 and 6). The results of the amino acid analysis of the samples were in agreement with the expected composition (Tables II and III)[10].

TABLE III

AMINO ACID ANALYSIS OF INSULIN B CHAIN, PRODUCED IN BACTERIA AND OF THE BOVINE B CHAIN STANDARD

Approximately 20 μg of bovine B chain and 20 μg of *E. coli* B chain purified twice by HPLC were hydrolysed and analysed in parallel.

Amino acid	*B chain from bacteria*		*Bovine B chain used as standard*	
	Found	*Predicted*	*Found*	*Predicted*
Aspartic acid	1.33	1	1.0	1
Threonine	1.99	2	1.03	1
Serine	1.25	1	1.04	1
Glutamic acid	2.92	3	2.84	3
Proline	0.91	1	1.01	1
Glycine	3.01	3	3.32	3
Alanine	1.03	1	2.01	2
Cisteine	N.D.*	2	N.D.*	2
Valine	2.85	3	2.81	3
Leucine	3.86	4	3.44	4
Tyrosine	1.78	2	1.83	2
Phenylalanine	2.64	3	2.86	3
Histidine	1.86	2	1.8	2
Lysine	0.91	1	0.82	1
Arginine	1.05	1	1.19	1

* N.D. = not determined

REFERENCES

1 Y. Aharonowitz and G. Cohen, *Sci. Am.*, 245 (1981) 66.
2 D. V. Goeddel, D. G. Kleid, F. Bolívar and H. L. Hyneker, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 106–110.
3 E. Gross, *Methods Enzymol.*, 11 (1967) 238–255.
4 X. Alvarado, O. Ladrón de Guevara, G. Estrada, L.Guereca, S. Antonio, N. Cruz and F. Bolívar, unpublished results.
5 T. W. Hearn and W. A. Hancock, *Biochem. Sci.*, 3 (1979) 58–62.
6 K. J. Wilson, A. Honegger and G. J. Hughes, *Biochem. J.*, 199 (1981) 43–51.
7 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979, 2nd ed., pp. 260–261.
8 G. Estrada, O. Ladrón de Guevara, X. Alvarado, S. Antonio, N. Cruz, L. Guereca and F. Bolívar, unpublished results.
9 W. J. Dixon and F. J. Massey, *Introduction to Statistical Analysis*, McGraw-Hill, New York, 1957.
10 A. L. Lehninger, *Biochemistry*, 2nd, ed., 1975, p. 109.