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COMPARISON OF CARBOXYMETHYL-CELLULOSE CATION-EXCHANGE CHROMATOGRAPHY AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE PURIFICATION OF GUINEA-PIG INSULIN FROM PANCREATIC EXTRACTS

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SUMMARY

Guinea-pig insulin was purified from pancreatic extracts either by carboxymethyl-cellulose cation-exchange chromatography with a sodium chloride gradient or by high-performance liquid chromatography (HPLC) on octadecyl silica with mixtures of acetonitrile and phosphate buffer. HPLC proved to be superior to ion-exchange chromatography in the purification of insulin with respect both to time saving and to the purity of the product.

INTRODUCTION

Highly purified insulin preparations are required for therapeutic use. Antigenicity of trace amounts of minor breakdown products may partly account for insulin antibodies which complicate the management of insulin-dependent diabetes[1,2].

Established methods of purifying insulin consist of the sequential use of gel and cation-exchange chromatography, the purity of the product being judged by polyacrylamide gel electrophoresis (PAGE)[3,4]. However, evidence is provided here the PAGE is not sensitive enough to demonstrate trace impurities, nor is it adequately selective to display the true heterogeneity of peptide mixtures since overlapping of protein bands occurs. Moreover, cation-exchange chromatography on carboxymethyl-cellulose is time consuming and not adequate for generating a single product.

Preliminary reports on the use of octadecyl silica (ODS) columns in reversed-phase high-performance liquid chromatography (HPLC) of mixtures of peptides purified by other methods, including commercial therapeutic insulin preparations[5,6], encouraged the use of HPLC in the present attempt to purify insulin from pancreatic extracts.

Guinea-pig insulin was the insulin of choice for this study not only because of its relevance to other aspects of our research, but also because of its atypic structure in comparison with the mammalian insulins, which helps to eliminate the analytical complications arising from polymerisation as pH rises above 2.0, especially in concentrated solutions[1].

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MATERIALS AND METHODS

Insulin extraction

The method used was based on those of Davoren[7] and Smith[8]. Guinea-pig pancreata (100 g) frozen in liquid nitrogen and stored at −70°C, were crushed in liquid nitrogen and extracted in two steps over 10 h at 3–4°C in a mixture of 74% ethanol (AnalaR) and 28% water (including tissue water), adjusted to pH 2.5 with orthophosphoric acid. Unrelated proteins were precipitated from the centrigued extract by adjusting the pH to 7.0 with ammonia solution (AnalaR, sp.gr. 0.91). After readjustment to pH 2.5 by concentrated hydrochloric acid (AnalaR, sp.gr. 1.18), the ethanol was distilled off on a rotary evaporator at 30°C. The lipid and protein slurry was removed by filtration and the aqueous concentrate was adjusted to 20% (w/v) with sodium chloride. The protein was precipitated with an equal volume of saturated picric acid, dissolved in acetone–water (4:1, v/v) and reprecipitated with ten volumes of acetone–hydrochloric (99:1, v/v). The resulting crude insulin was extracted twice with acetone and once with diethyl ether and dried in air.

Gel filtration

A 1.5 × 80 cm Sephadex G-50 superfine column (Pharmacia, Uppsala, Sweden) was equilibrated and run with 1.0 mol/l acetic acid at 4°C, at a rate of 5 ml/h. One-ml fractions were collected.

Cation-exchange chromatography

A 0.9 × 30 cm column (Pharmacia) packed with carboxymethyl-cellulose, CM 52 (Whatman, Maidstone, Great Britain) was equilibrated with 0.04 mol/l sodium dihydrogen citrate (pH 3.3) at 4°C. A linear gradient of sodium chloride (0–0.18 mol/l) as indicated in the Results and discussion and the legend to Fig. 1 was applied at variable rates. Three-ml fractions were collected.

HPLC

A Varian 5000 liquid chromatograph with UV 50 variable-wavelength detector (set at 230 nm) was used, with a 0.5 × 25 cm column of octadecyl silica ODS-C_{18} (Shandon Southern, Runcorn, Great Britain). The mobile phase consisted of a binary mixture of acetonitrile and 0.4 mol/l phosphate solution (pH 2.4) of varying proportions. The protein peaks were pooled, freeze dried and desalted by picrate precipitation, reprecipitated by acetone–hydrochloric acid and dried, as in the last preparative steps of insulin extraction described above. A guinea-pig insulin preparation (Novo Research Institute, Bagsvaerd, Denmark) was used as a marker after separation by HPLC from the albumin in the preparation.

Polyacrylamide gel electrophoresis

The running gel was prepared in 75 × 5 mm columns with 7.5 g of acrylamide, 50 mg ammonium persulphate (BDH, Poole, Great Britain), 200 μl ethylenediacrylate (Serva, Heidelberg, G.F.R.) and 50 μl N,N,N′,N′-tetramethylethylenediamine (TMED) (Sigma London, Poole, Great Britain) in 100 ml of 0.38 mol/l Tris–HCl buffer (pH 8.9). The spacer gel (0.2 ml per tube) was prepared with 1.9 g cyanogum, 50 mg ammonium persulphate (BDH), 115 μl ethylenediacrylate and 50 μl TMED in

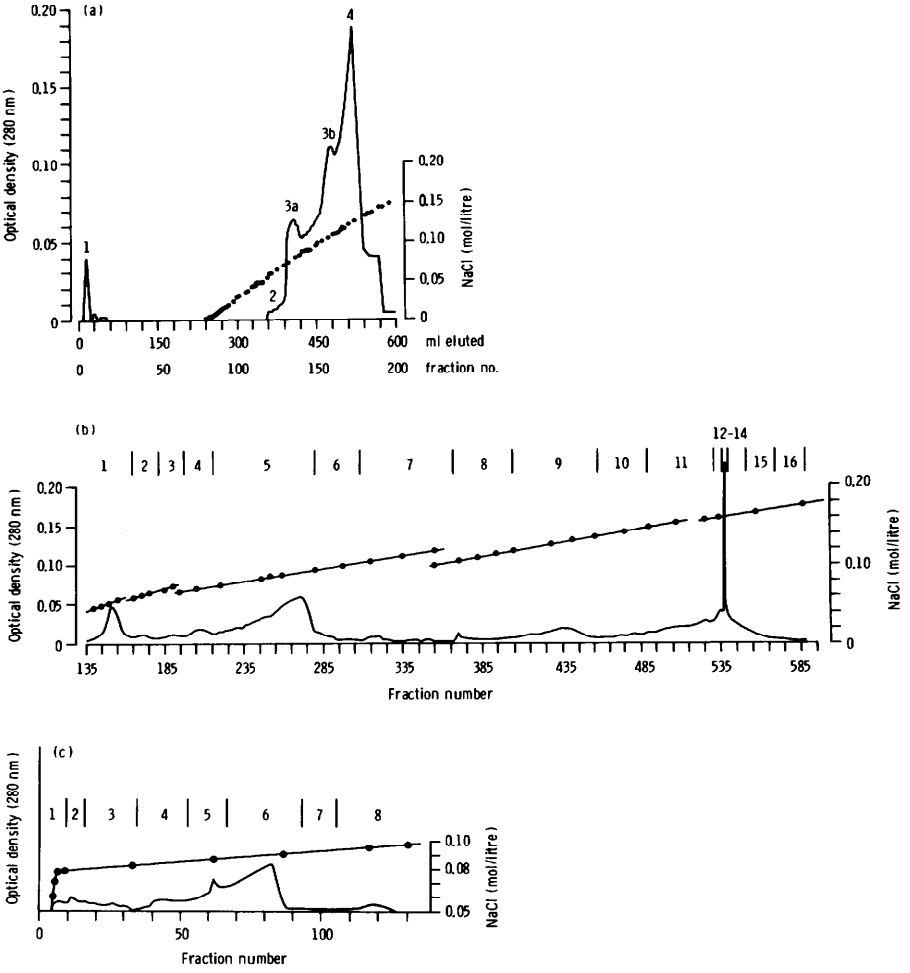

Fig. 1. Carboxymethyl-cellulose cation-exchange chromatography of crude pancreatic extract containing insulin. a, NaCl gradient (0.00–0.15 mol/l) applied over 43 h; b, NaCl gradient (0.00–0.018 mol/l) applied over 175 h; c, fraction 5 was rechromatographed with a NaCl gradient of 0.05–0.10 mol/l.

50 ml of 0.062 mol/l Tris–HCl buffer, pH 6.7. The electrode buffer was 0.01 mol/l Tris–glycine, pH 8.3. Electrophoretic runs were carried out at a constant current of 2.5 mA per column for 4 h.

Blood glucose was estimated by the method of Trinder[9].

RESULTS AND DISCUSSION

When ion-exchange chromatography was carried out at a rate of 8 ml/h (43 h for the NaCl gradient, 74 h for the entire chromatography) (Fig. 1a), the protein peaks were not sufficiently resolved. On electrophoresis (Fig. 2a), bands with the electrophoretic mobility of guinea-pig insulin (Novo) were seen throughout the range of 0.047–0.145 mol/l NaCl.

When the fraction pools denoted as 2, 3a, 3b and 4 in Fig. 1a were chromatographed on Sephadex gel (Fig. 3a, b and c), protein bands with insulin-like electrophoretic mobility were observed in the major protein peaks eluted from these fractions. HPLC of the protein of these peaks with 22–29% acetonitrile in 0.4 mol/l phosphate (pH 2.4) revealed greater heterogeneity than was visible on PAGE columns (Table Ia).

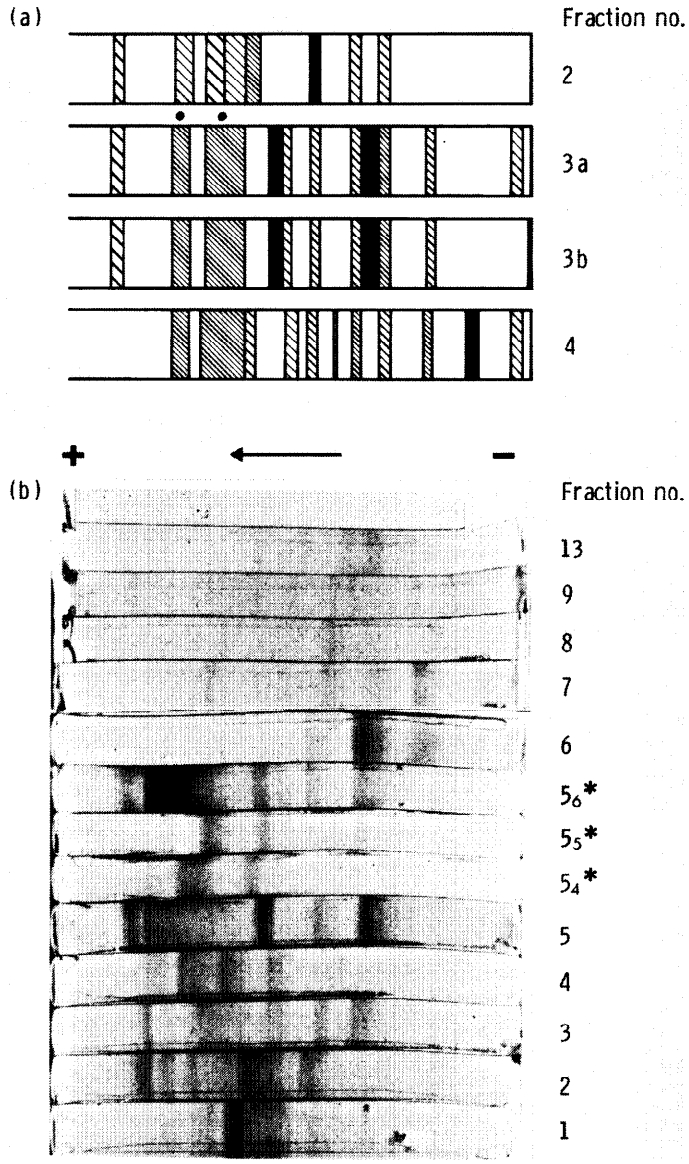

Fig. 2. Polyacrylamide gel electrophoresis of the CM-cellulose cation-exchange chromatographic fractions of guinea-pig pancratic extracts a, After chromatography, as shown in Fig. 1a; bands corresponding to Novo guinea-pig insulin are indicated by the dots alongside the gel. b, After chromatography as shown in Fig. 1b and c. The fractions marked* correspond to nos. 4, 5 and 6 in Fig. 1c, which were derived by rechromatography of fraction 5 (Fig. 1b).

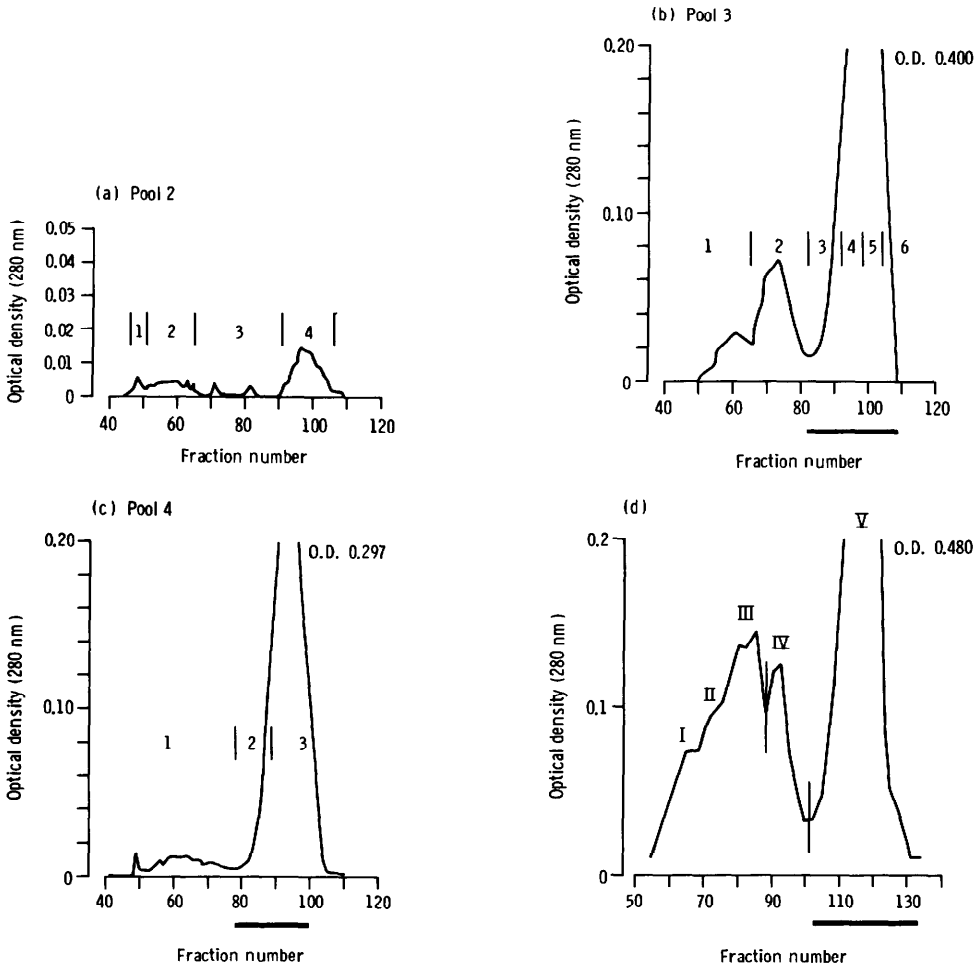

Fig. 3. Sephadex G-50 gel chromatography of pancreatic extracts. a–c, Gel separation of proteins in pools 2–4, respectively, of Fig. 1a; d, gel chromatography of extract without previous cation exchange chromatography. The fractions containing insulin are indicated with a line under the curve.

In the extended cation-exchange chromatography of the crude pancreatic extract, protein elution started at 0.04 mol/l NaCl and ended at 0.18 mol/l NaCl over 175 h (Fig. 1b). The asymmetric peak eluting in the 0.074–0.094 mol/l range of the NaCl gradient, 5 on Fig. 1b, was rechromatographed using 0.078–0.096 mol/l NaCl (Fig. 1c). The protein elution profile was very similar to that observed for porcine insulin on CM-cellulose columns at pH 5.38 by Dillon and Romans[10]. These workers demonstrated that their asymmetric protein peak (corresponding to fractions 4, 5 and 6 in Fig. 1c) consisted of interconvertible forms of purified porcine insulin. Even though guinea-pig insulin does not polymerise[1], the PAGE of these fractions 4, 5 and 6 gave at least six protein bands (Fig. 2b). Not enough material was available after the second CM 52 chromatography to carry out gel chromatography for separating these proteins according to size. However, the HPLC profiles of these fractions (*i.e.*, 1–7,

TABLE I

HPLC CHARACTERIZATION OF PROTEIN PEAKS IN GUINEA-PIG PANCREATIC EXTRACTS PURIFIED BY SEPHADEX G-50 AND/OR CM-CELLULOSE CATION-EXCHANGE CHROMATOGRAPHY

Fractions a are Sephadex G-50 fractions after cation-exchange chromatography of the extracts as shown in Figs. 2b, c, 3a and 4a; b are fractions after cation-exchange chromatography only as shown in Fig. 4b and c, PAGE profiles of which are in Fig. 3b; c are fractions after Sephadex G-50 chromatography only, shown in Figs. 2d, 5a and b, gel profiles of which are summarised in Fig. 6.

	Chromatography fraction No.	*Eluting NaCl gradient (mol/l)*	*HPLC peak No.*														
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*11*	*12*	*13*	*14*	*15*	*16*	*17*
a	3_1	0.070–0.110						↑	+				+	+				
	3_2	0.070–0.110		+	↑				+			+						
	3_3	0.070–0.110	+				+	+	↑	+	+	+		↑				
	3_4	0.070–0.110	↑				+	+	↑	↑	↑	↑	↑	↑				+
	3_5	0.070–0.110	↑				↑	+	↑	↑	↑	↑						↑
	3_6	0.070–0.110	↑				↑	+	↑	↑	↑	↑						↑
	4_2	0.120–0.135	+				+	↓	↑	↑	↑		+	+	+			
	4_3	0.120–0.135	+				↑	↓	↑	↑	↑		+	+	↑			↑
b	5_4	0.082–0.085	+	+	+	+		+	↑	↑	↑	↑	+				+	
	5_5	0.085–0.087		+	+	+		+	↑	↑	↑	+	+				↓	
	5_6	0.087–0.091		+	+	+		+	↑	↑	↑	+	+	+				+
	6	0.094–0.102		↑	↑	↑							+	+	↑	↑	↑	↑
	7	0.102–0.118		↓	+	↑	+						+					↑
c	IV	–																
	V	–	+	+	+	+	+	+	↑	↑	+	↑	+	+	+	+	+	↑

* These HPLC protein peaks are shown in Fig. 5b: +, the detection of a particular peak; ↑, an increase in the optical density reading of a particular peak; ↓, a decrease in the optical density of a particular peak.

Fig. 1b) and of the subfractions 4–6 (Fig. 1c) obtained by the slow cation-exchange chromatography of crude insulin could be compared to the HPLC profile of the subfractions associated with the "insulin" peak of G-50 gel chromatography of crude insulin (Fig. 3b and c). Three separate profiles obtained by a discontinuous gradient of 15–30% acetonitrile in phosphate buffer (pH 2.4) are shown in Fig. 4. The proteins of the "insulin" peak (Fig. 4a) match those eluted from CM-cellulose over the 0.082–0.091 mol/l NaCl gradient (Fig. 4b) but not the proteins of the fractions eluted with NaCl in excess of 0.094 mol/l (Fig. 4c; *cf.*, Table I).

When crude insulin was separated by G-50 gel chromatography from pancreatic extracts not previously subjected to ion-exchange chromatography (Fig. 3d), the HPLC profile of the "insulin peak" only (*i.e.*, peak V, Fig. 3d) but not of others (*e.g.*, peak IV) over the 15–30% acetonitrile gradient in 0.4 mol/l phosphate (pH 2.4) matched those in Fig. 4a and b. The HPLC profiles of peak IV and peak V are shown in Fig. 5a b, respectively, and the mathcing of the proteins in peak V with those in the profiles given in Fig. 4a and b is summarised in Table I. Comparison of the results in Table Ib with those in a and c indicates that the protein heterogeneity of the product was not sufficiently improved by the slow cation exchange chromatography. The electrophoretic characterisation of the individual peaks in fractions IV and V (Table I

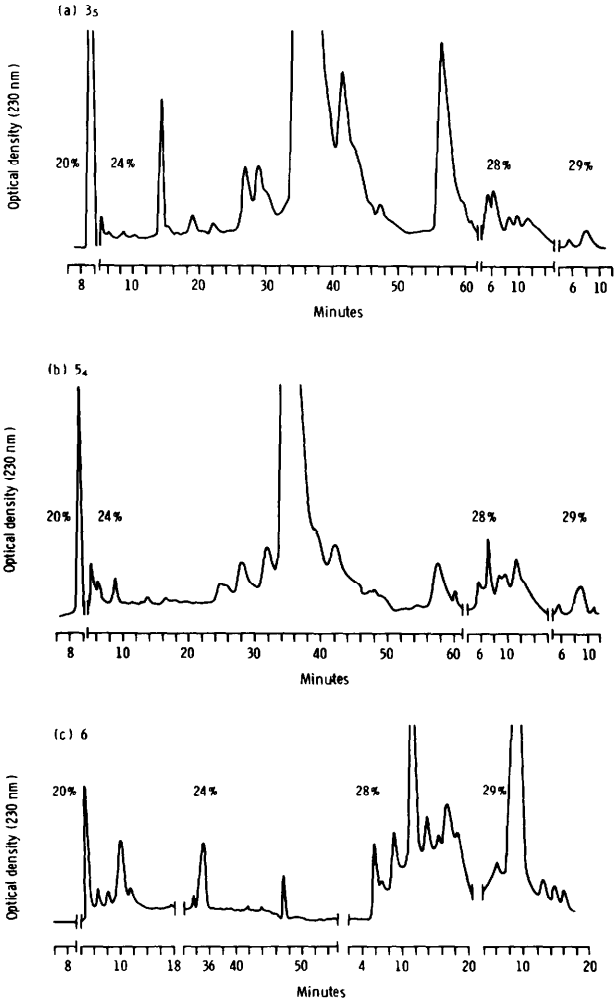

Fig. 4. HPLC of the protein subfractions obtained by cation-exchange chromatography of crude insulin. a, Subfraction no 5 shown in Fig. 2b; b, subfraction no. 4 shown in Fig. 1c; c, subfraction no 6. shown in Fig. 1b. The percentage figures indicate the changes of the acetonitrile composition of the binary mixture of acetonitrile–0.4 mol/l phosphate (pH 2.4). Optical density range was 0.00–0.05 and comparable quantities of protein were injected in each case.

and Fig. 5) is summarised in Fig. 6 indicating the limited selectivity of the PAGE system used and the consequent overlapping of protein bands.

Because of the limited availability of the separated protein fractions, extensive investigation of their biological activity was not possible. However single subcutaneous injections of 1.5 mg protein per mouse (using duplicate mice) of the protein from peak 7, or fraction 3_3 (Table I) were equally effective in reducing the blood glucose concentration by 1–2 mmol/l within 20–25 min and rendered dormant these otherwise highly active mice. Mice injected with other proteins from fractions 6, 7, 13 of Fig. 1b) remained active, and the blood glucose concentrations were not altered.

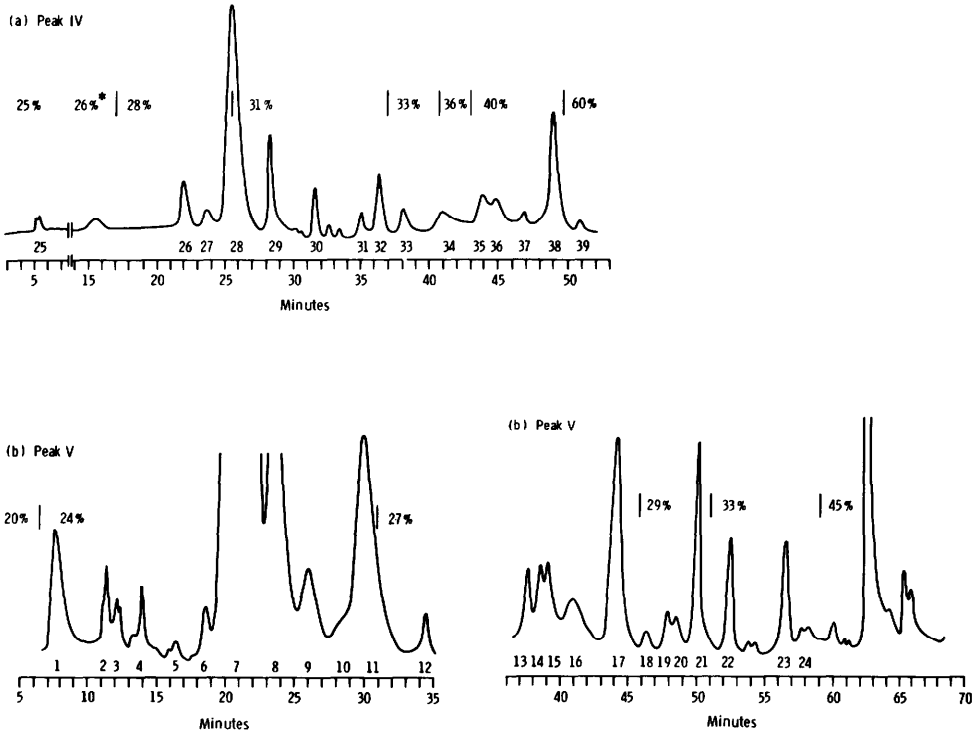

Fig. 5. HPLC of pooled fractions IV and V obtained by Sephadex G-50 gel chromatography of crude insulin (as in Fig. 3d) Peak V contained insulin and peak IV probably proinsulin. The peptide profile of peak V was more detailed and is presented in two parts. The percentage figures indicate the changes of the acetonitrile composition of the binary mixture of acetonitrile–0.4 mol/l phosphate (pH 2.4). Optical density range was 0.00–0.05 and comparable quantities of protein were injected in each case.

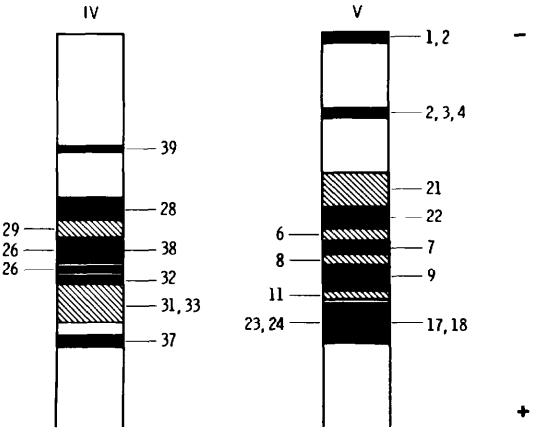

Fig. 6. A superimposed summary of the polyacrylamide gel electrophoresis of the individual protein peaks separated by HPLC of peaks IV and V shown in Fig. 5a and b. The numbers against the bands are as indicated in Table I.

The binding of insulin by receptors *in vivo* or *in vitro* is not completely understood. Interfering effects of altered insulins, or of fragments of insulins, on the binding and breakdown of intact mammalian insulins by cell preparations have been reported[1,2]. It is difficult to explain the effects *in vivo* in the mouse of a small amount of a greatly mutated insulin such as the guinea-pig insulin. Clearly there is a need for definite identification of these proteins by means of amino acid analysis as well as biological tests.

Although the method appears applicable to a crude unprocessed pancreatic extract, it is more economical to carry out a preliminary gel chromatography of the extract to separate the insulin and proinsulin containing fractions which allows for rapid HPLC purification of both proteins separately (see ref. 11).

CONCLUSIONS

This work was carried out with two objectives; (i) to use HPLC for preparative purification of insulin from pancreatic extracts subjected to minimal preliminary processing; (ii) to compare the efficiency of this approach with that of CM-cellulose cation-exchange chromatography.

It was found that HPLC was simpler and much more rapid in the preparative yield of pure products, and potentially very useful for studying peptide heterogeneity, as compared to CM-cellulose cation-exchange chromatography or PAGE, especially in the identification of persistent trace contaminants of therapeutic preparations of peptide hormones (ref. 11), probably not always detectable by the isocratic HPLC of these preparations as hitherto reported in the literature[5,6,12].

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