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Note

Reversed-phase high-performance liquid chromatography of insulins from different species*

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The use of large pore silicas (300 Å and 500 Å) adequately derivatized and end capped for reversed-phase high-performance liquid chromatography (RP-HPLC) has made the resolution and recovery of large peptides and proteins not only possible, but also considerably more efficient and rapid[1–8] than such other methods as gel permeation, partition chromatography, or electrophoresis. Furthermore, whereas separation of small proteins on conventional RP-HPLC supports (80–120 Å silicas) could be achieved after optimization of different chromatographic parameters (solvent selection and gradient shape[9–13], alkyl chain length[12], flow-rate[6,11], temperature[11,12], origin of the silica[9], etc.) it is found that larger pore silicas are probably less descriminating and will allow for overall better recoveries than the smaller-pore supports whatever parameters are being investigated[6–8]. It is thought[1,2,5,6] that wider pore material is better able to permit free access of the large size proteins into the porous matrix and better partition across phases.

While insulin and its A and B chains have often been used as models for evaluating chromatographic systems[9,11] only a few reports can be found in the literature that describe the separation of insulin from its potential by-products[14–17].

Only two reports can be found showing the separation of insulins from different species: Tsune *et al.*[18] separated ovine, bovine, porcine, and equine insulins, while Chance *et al.*[17] separated bovine, ovine, porcine and human insulins in their study of the characteristics of biosynthetic human insulin.

We wish to report here the separation of eight different insulins using (330 Å pore size) columns that have been derivatized three ways (C_{18}, phenyl and C_4) and using two buffer systems [0.1 trifluoroacetic acid (TFA) in acetonitrile and triethylammonium phosphate pH 2.25 (TEAP 2.25)–acetonitrile]. The use of C_4, phenyl and diphenyl (not reported here) columns has not been extensive and is relatively new to the separation of peptides and proteins[7,8]. Their use, however, played a major role in the isolation of rat hypothalamic growth hormone and corticotropin releasing factors[7,8,19], as well as salmon gonadotropin releasing factor[20] and human pancreatic growth hormone releasing factor[7].

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MATERIALS AND METHODS

Apparatus

The apparatus consists of Waters Assoc. models: two 6000A pumps; data module, recorder and integrator; Wisp 710B, automated sample injector; System Controller 720, programmer. The detector was a Perkin-Elmer LC-75 variable-wavelength UV–visible spectrophotometer.

Buffers

The TEAP buffer at pH 2.25 was made by bringing the pH of 0.25 *N* phosphoric acid to 2.25 with triethylamine distilled over *p*-toluenesulfonylchloride. The buffer was then filtered through a C_{18} cartridge in a Waters Assoc. Prep LC-500 to remove hydrophobic impurities. The aqueous "A" buffer was TEAP 2.25. The organic "B" buffer was: A buffer–Burdick and Jackson distilled-in-glass acetonitrile (40:60).

The 0.1% TFA buffer was made with 0.45-μm-filtered deionized water and distilled trifluoroacetic acid. The aqueous, A, buffer was 1.00 ml of TFA in 1.00 l of deionized water and the organic, B, buffer consisted of 1.00 ml of TFA in 400 ml of deionized water made to the mark of a 1.00-l volumetric flask with acetonitrile.

Columns

All columns were reversed-phased, 25 × 0.46 cm, 330 Å, 5 μm particle size Vydac (Separations Group, Hesperia, CA, U.S.A.). Column number V-1830-4 was packed with C_4, end-capped material, column number V-62482-1 was packed with phenyl, end-capped material and column number V-1820-27 was packed with C_{18} end-capped material.

Insulins

The insulins were the generous gift of Dr. R. Chance of the Lilly Research Labs. Crystalline bovine insulin, lot 615-70N-80; crystalline porcine insulin. lot 615-075-256; pancreatic human insulin, lot 615-1054B-214-1; chicken insulin, lot 615-1082B-249; ovine insulin, lot 615-1112B-108-I; rabbit insulin lot 615-D63-29-C; rat insulin (I and II), lot 615-845-138; and rat insulin I, lot 615-D63-13-C. The structure of the insulins were from Dayhoff[21] (Table I).

Peak identification

The peaks were identified by adding an amount of known insulin to a stock solution of the mixed insulins, *i.e.* "spiking" the standards.

RESULTS AND DISCUSSION

Results for the separations of the insulins on the different columns (C_4, phenyl and C_{18}) using identical gradients and 0.1% TFA buffer are given in Table II. Except for the inability of the phenyl column to separate ovine and rabbit insulins, even though significant difference in substitutions occur at four different positions (see Table I) the six insulins selected elute in the same order on these columns. The last three insulins, (rabbit, human and porcine), separate however somewhat better on

TABLE I

HUMAN INSULIN

A-Chain: H-Gly-Ile-Val-Glu-Gln-Cys-Cys-Thr-Ser-Ile-Cys-Ser-Leu-Tyr-Gln-Leu-Glu-Asn-Tyr-Cys-Asn-OH

B-Chain: H-Phe-Val-Asn-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH

Source	*Modifications of the insulin structure*										
	A. Chain Position				*B. Chain Position*						
	4	*8*	*9*	*10*	*1*	*2*	*3*	*9*	*27*	*29*	*30*
1 Chicken	Glu	His	Asn	Thr	Ala	Ala	Asn	Ser	Ser	Lys	Ala
2 Bovine	Glu	Ala	Ser	Val	Phe	Val	Asn	Ser	Thr	Lys	Ala
3 Ovine	Glu	Ala	Glu	Val	Phe	Val	Asn	Ser	Thr	Lys	Ala
4 Rabbit	Glu	Thr	Ser	Ile	Phe	Val	Asn	Ser	Thr	Lys	Ser
5 Human	Glu	Thr	Ser	Ile	Phe	Val	Asn	Ser	Thr	Lys	Thr
6 Porcine	Glu	Thr	Ser	Ile	Phe	Val	Asn	Ser	Thr	Lys	Ala
7 Rat I	Asp	Thr	Ser	Ile	Phe	Val	Lys	Pro	Thr	Lys	Ser
8 Rat II	Asp	Thr	Ser	Ile	Phe	Val	Lys	Ser	Thr	Met	Ser

the phenyl column than on either aliphatic column. Although this effect is not dramatic with the insulins, this has been used to great advantage in the separation of synthetic peptides*. It can be seen that the C_{18} column is the most hydrophobic and that the phenyl column is the least hydrophobic, there being a small but noticeable difference between the phenyl and C_4 column. Pearson and Regnier[23] found that in a TFA 2-propanol system, the retention times were similar for chain lengths from C_2 to C_{22} and propose that the proteins used in their studies, of which insulin was one, "...only interact with the extreme top portion of alkyl chains...". Our findings of different retention times for the C_4 and C_{18} columns using a TFA–acetonitrile buffer warrant further investigation into the interaction between the organic modifier (acetonitrile *vs.* propanol) and the reversed-phase support. In the TEAP system (Table III, Figs. 1–3), the elution order is the same and the phenyl column is still unable to resolve ovine and rabbit insulin. There is, however, a striking change in the comparative hydrophobicities of the columns. The retention times on the phenyl and C_4 columns are nearly identical, and the retention times on the C_{18} columns are much greater.

The separation of eight insulins on a C_4 column in 0.1% TFA is illustrated in Fig. 4 and a 0.1% TFA separation is compared to a TEAP 2.25 separation in Table IV. This is remarkable, but not unexpected (see separation of β-endorphins[11], when

* When purifying synthetic peptides on a large scale using Vydac (15–20 μm, C_{18}, C_4 or phenyl) packed cartridges fitting Waters Assoc. Prep LC-500, we have found that dramatic differences in selectivity existed between those supports. Peptides rich in aromatic residues have been shown, for example, to exhibit higher affinity for phenyl columns than C_{18} (ref. 22), thus leading us to believe that stacking of peptidic aromatic residues and those on the support may be an element of the retention mechanism.

TABLE II

C_4 *vs.* PHENYL *vs.* C_{18} IN 0.1% TFA

Gradient: from 40 to 60% B in 25 min; flow-rate: 2 ml/min. Buffer A: 0.1% TFA in water; buffer B: 0.1% TFA in water–acetonitrile (40:60).

	Retention time (min)		
	C_4	*Phenyl*	C_{18}
Chicken insulin	14.31	13.20	15.46
Bovine insulin	17.81	17.38	18.81
Ovine insulin	18.23	17.70	19.26
Rabbit insulin	18.60	17.70	19.76
Human insulin	18.85	17.86	20.01
Porcine insulin	19.16	18.36	20.35

one considers that rabbit and human insulins differ by a methyl group only, *i.e.*, the substitution of threonine for serine at position B30, Table I). In general, under identical gradient conditions, elution of insulins with TEAP resulted in lower capacity factors and better separation though it should be noted that the separation of human insulin from rabbit insulin is greater in 0.1% TFA than in TEAP. The peak shapes of Rat I and Rat II insulin are already broad in 0.1% TFA. This problem is even greater in the TEAP buffer and is thought to be due to the slower exchange kinetics of the solute interacting through the "bilayer" of triethylamine phosphate compared to that in 0.1% TFA[24].

All three columns and both buffers can be utilized for effective separation of insulins. TEAP 2.25 generally allows for greater separation and quicker analysis but some compounds chromatograph more broadly. The 0.1% TFA buffer is not as selective but gives better peak shape on the more hydrophobic insulins. The C_{18} column shows greater resolution of the more hydrophilic insulins. The phenyl column does not show as much resolution overall, yet porcine and human insulins separated best on that column in TFA.

It should therefore be reiterated that although the large-pore C_4 column in

TABLE III

C_4 *vs.* PHENYL *vs.* C_{18} IN TEAP 2.25

Gradient: from 40% to 48% B in 25 min; flow-rate: 2 ml/min. Buffer A: TEAP 2.25; buffer B: TEAP 2.25–acetonitrile (40:60).

	Retention time (min)		
	C_4 *(Fig. 1)*	*Phenyl (Fig. 2)*	C_{18} *(Fig. 3)*
Bovine insulin	9.63	9.82	20.88
Ovine insulin	10.30	10.35	22.00
Rabbit insulin	10.50	10.35	22.36
Human pancreatic insulin	10.90	10.82	22.80
Porcine insulin	11.26	11.25	23.22

TABLE IV

0.1% TFA *vs.* TEAP 2.25 ON A C_4 COLUMN

Gradient: from 30 to 55% B in 25 min; flow-rate: 2 ml/min, Buffers as defined in Table II (TFA) or Table III (TEAP).

	Retention time (min)	
	0.1% TFA	*TEAP 2.25*
Chicken insulin	15.86	14.68
Bovine insulin	18.53	17.72
Ovine insulin	18.93	18.25
Rabbit insulin	19.11	18.55
Human insulin	19.58	18.80
Porcine insulin	19.70	19.06
Rat I insulin	19.81	19.61 (Broad)
Rat II insulin	20.90	21.03 (Broad)

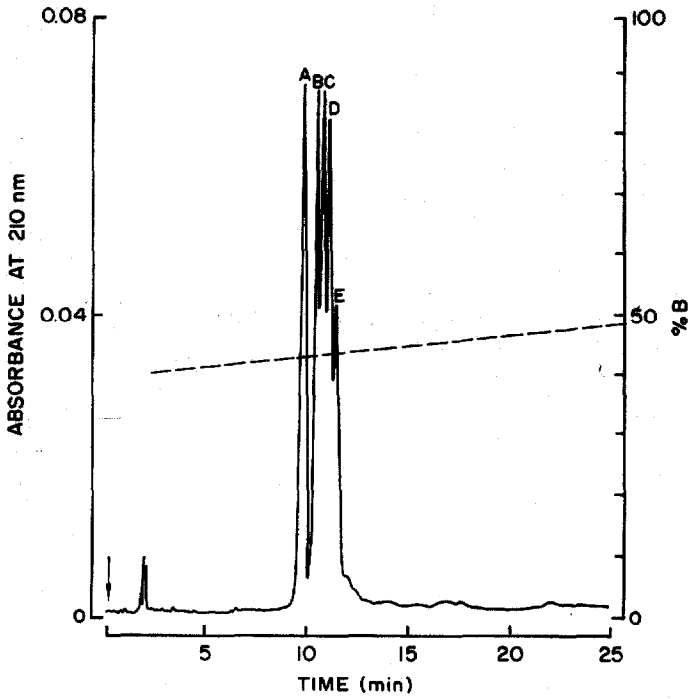

Fig. 1. Separation of A, bovine insulin, 9.63 min; B, ovine insulin, 10.30 min; C, rabbit insulin, 10.50 min; D, human insulin, 10.90 min; E, porcine insulin, 11.26 min. Column: V-1830-4, Vydac C_4. Load: 5 μg each. Flow-rate: 2 ml/min. Pressure: 2900 p.s.i. A: TEAP 2.25. B: Acetonitrile–A (60:40). Gradient: from 40% to 48% B in 25 min. Detection: 0.08 a.u.f.s./210 nm.

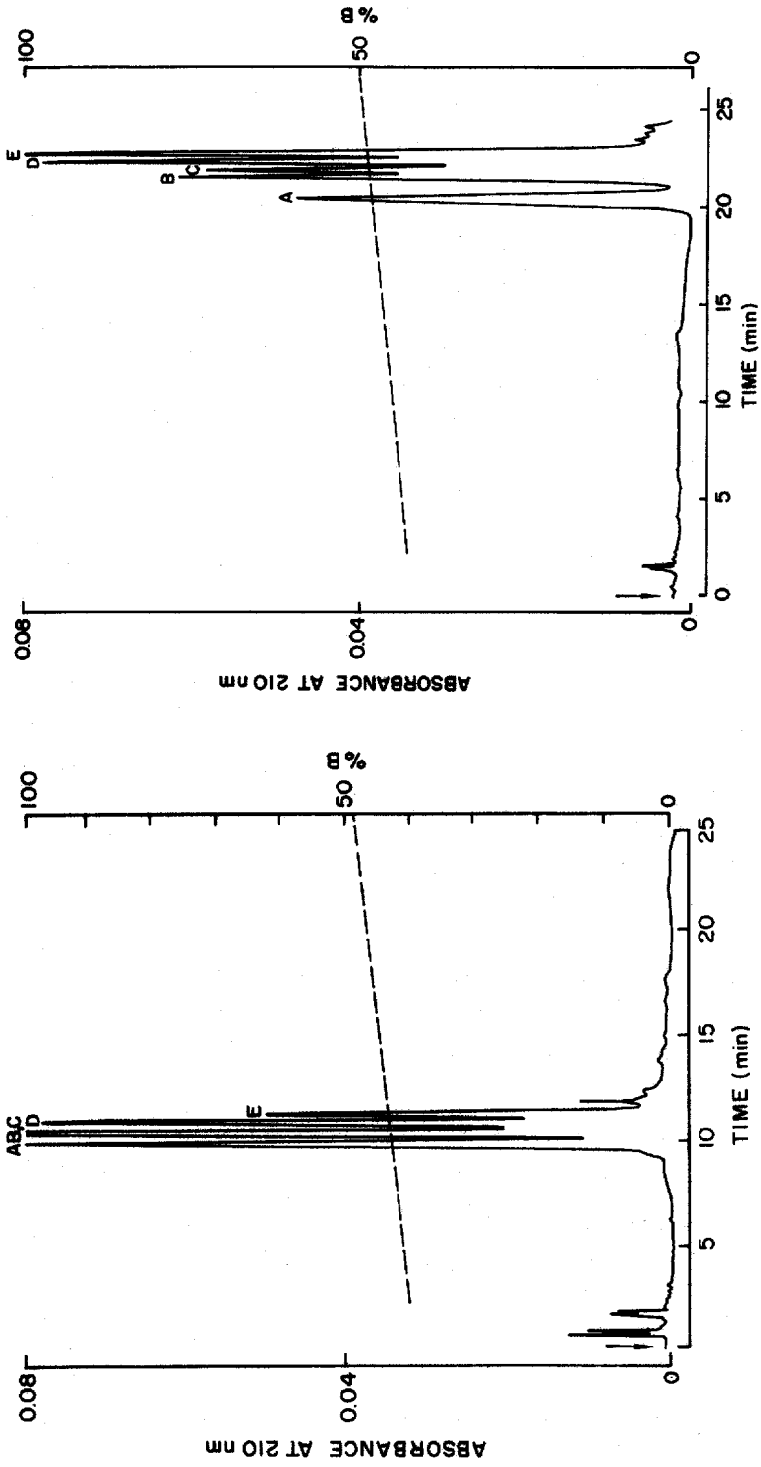

Fig. 2. Separation of A, bovine insulin, 9.82 min; B, ovine insulin, 10.35 min; C, rabbit insulin, 10.35 min; D, human insulin, 10.82 min; E, porcine insulin, 11.25 min. Column V-62482-1 Vydac phenyl. Load: 5 μg each. Flow-rate: 2 ml/min. Pressure: 2600 p.s.i. Buffers, gradient and detection as in Fig. 1.

Fig. 3. Separation of A, bovine insulin, 20.88 min; B, ovine insulin, 22.00 min; C, rabbit insulin, 22.36 min; D, human insulin, 22.80 min; E, porcine insulin, 23.22 min. Column: V-1820-27, Vydac C_{18}. Load: 5 μg each. Flow-rate: 2 ml/min. Pressure: 3200 p.s.i. Buffers, gradient and detection as in Fig. 1.

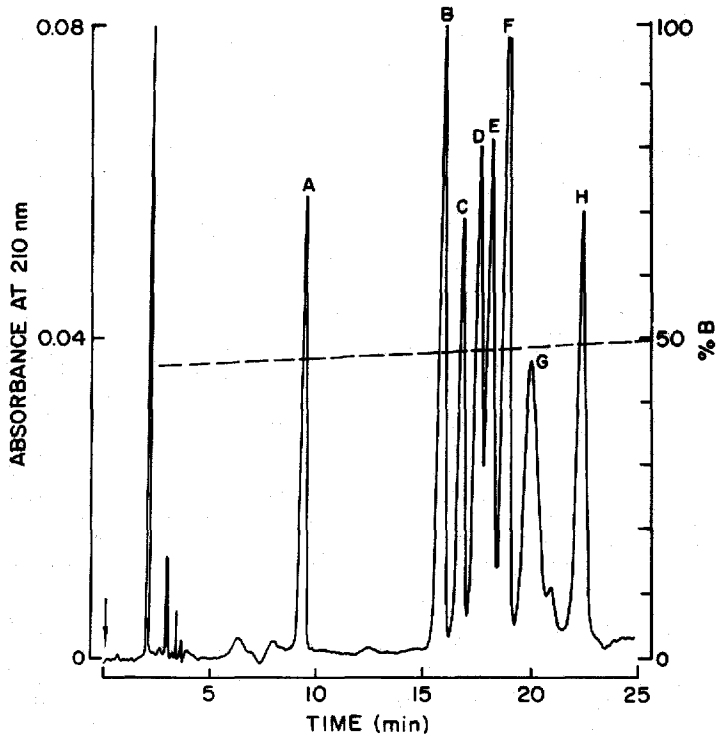

Fig. 4. Separation of A, chicken insulin, 9.23 min; B, bovine insulin, 15.70 min; C, ovine insulin 16.66 min; D, rabbit insulin, 17.43 min; E, human insulin, 18.00 min; F, porcine insulin, 18.76 min; G, rat I insulin, 19.96 min; H, rat II insulin 22.36 min. Column: V-1830-4, Vydac C_4. Load: 5 μg each. Flow-rate 2 ml/min. Pressure: 2900 p.s.i. A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile–water (60:40). Gradient: from 45% to 50% B in 25 min. Detection: 0.08 a.u.f.s./210 nm.

TFA separated the insulins quickly and effectively with minimization of such unexplained phenomena as "memory effect" often observed with smaller-pore silicas, another combination of column and buffer might give better results in detecting the minor impurities of each insulin or in separating selected insulins.

CONCLUSION

From these studies we could conclude that as a function of their ion pairing capacity, mobile phases may have significantly more drastic effects on a given separation than the particular bonded phases. We have found that the supports and mobile phases reported here are compatible and their usage results in most remarkable separations.

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