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SEPARATION OF LARGE POLYPEPTIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The separation of polypeptides of biological interest has been investigated by hydrophobic chromatography. It was found that both the eluent composition and the salinity of the eluent have a profound influence on the efficiency and selectivity of the separation, and the use of isopropanol as organic modifier in the eluent improved the selectivity. The expected retention times were calculated for ACTH derivatives and a good correlation between the predicted and found capacity factors was obtained. The applicability of the method is demonstrated for samples of oxytocin, $ACTH_{1-32}$, aprotinine and insulins.

INTRODUCTION

In the last few years great efforts have been made to improve the efficiency and selectivity of the high-performance liquid chromatography (HPLC) methods used for the separation of proteins and polypeptides. Increasing numbers of individual polypeptide separations are now being published[1–13] (for a comprehensive review see ref. 14). Most of the methods published so far can be devided into two groups.

In the first group, the separation mechanism proceeds according to liquid–liquid partition and the retention is mainly dependent on the hydrophobic nature of the compounds investigated[15–17]. The hydrophobicity of polypeptides can be controlled by the pH or the salt and organic modifier concentrations of the eluent. In most cases, a gradient elution technique was for the most hydrophobic compounds, by increasing the organic solvent concentration in the mobile phase. A very elegant gradient elution system has been described by O'Hare and Nice[12] for the separation of proteins and polypeptides. On the basis of their retention data, the retention coefficients of amino acid residues were calculated by Meek[13], enabling the prediction of retention times of different polypeptides.

The second group comprises reversed-phase ion-pair chromatographic methods, which employ a chemically bonded non-polar stationary phase and a mixture of organic solvent (mainly acetonitrile or methanol) and buffered water containing a small amount of a anionic or cationic ion-pairing reagent as eluent. Although a wide variety of such methods have been reported for polypeptide separation[4,5,8,10,11],

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sometimes the ion-pair formation seems to be questionable, identical retentions being obtained for the same compounds in the presence and in the absence of ion-pair reagents[10].

The main aim of our work was to find an isocratic separation system for some polypeptides manufactured at Gedeon Richter (Budapest, Hungary), as well as to study the effects of the type and concentration of the organic modifier and that of salt concentration on the efficiency and selectivity of the separation.

EXPERIMENTAL

A Hewlett-Packard 1081/A liquid chromatograph equipped with a loop injector and variable-wavelength UV-detector (Schoeffel Model 770) was used. The separations were performed on a prepacked Nucleosil 10 C_{18} column, 250 × 4,6 mm I.D. (Chrompack, Middelburg, The Netherlands) using the following eluent systems with a flow-rate of 1 ml/min (only the eluents found to be optimal are listed):

Eluent A: solutions 1 and 2 (4:6). Solution 1 contains 16,7% acetonitrile in methanol; solution 2 contains 0.1 *M* sodium sulphate in 0,01 *M* aqueous phosphate buffer, pH 2.2.

Eluent B: solutions 1 and 2 (35:65).

Eluent C: solutions 1 and 2 (6:4).

Eluent D: solutions 1 and 3 (3:7). Solution 3 contains 0,05 *M* sodium sulphate in 0,01 *M* aqueous phosphate buffer, pH 6.5.

Eluent E: solutions 4 and 5 (35:65). Solution 4 contains 16.7% isopropanol in methanol; solution 5 contains 0.1 *M* sodium perchlorate in 0.01 *M* aqueous phosphate buffer, pH 2.2.

Eluent F: solutions 6 and 5 (4:6). Solution 6 contains 33.3% isopropanol in acetonitrile.

All solvents used were of HPLC grade and were obtained from E. Merck (Darmstadt, G.F.R.). The chemicals were of analytical grade (Reanal, Budapest, Hungary). Most of the compounds investigated were prepared at Gedeon Richter and their quality was monitored by HPLC and other analytical methods.

RESULTS AND DISCUSSION

From the separation possibilities mentioned above, hydrophobic chromatography according to Molnár and Horváth[16] was chosen for our experiments. In this case the retention can be controlled by ionization of the solutes. To suppress the dissociation of the ionizable functional groups of polypeptides, a high salt concentration and low pH in the eluent are employed, resulting in good peak shape and reproducible retention.

The capacity ratios, k, of polypeptides are generally very sensitive to the concentration of the organic modifier. However, only a small difference in selectivity can be achieved by changing the organic solvents[12,18]. For this reason, methanol and acetonitrile are generally accepted solvents in the HPLC analysis of polypeptides.

In order to develop an isocratic chromatographic method for some polypeptides of biological interest, various eluent compositions containing relatively high amounts of inorganic salts (sodium sulphate and sodium perchlorate) were studied. It

was found that the use of 0.1 *M* salt concentrations in the eluent gave a good peak shape and better resolution compared to eluents which do not contain salts. This is illustrated in Fig. 1 for the separation of bovine and porcine insulins and desamido-insulins in the presence and in the absence of sodium sulphate.

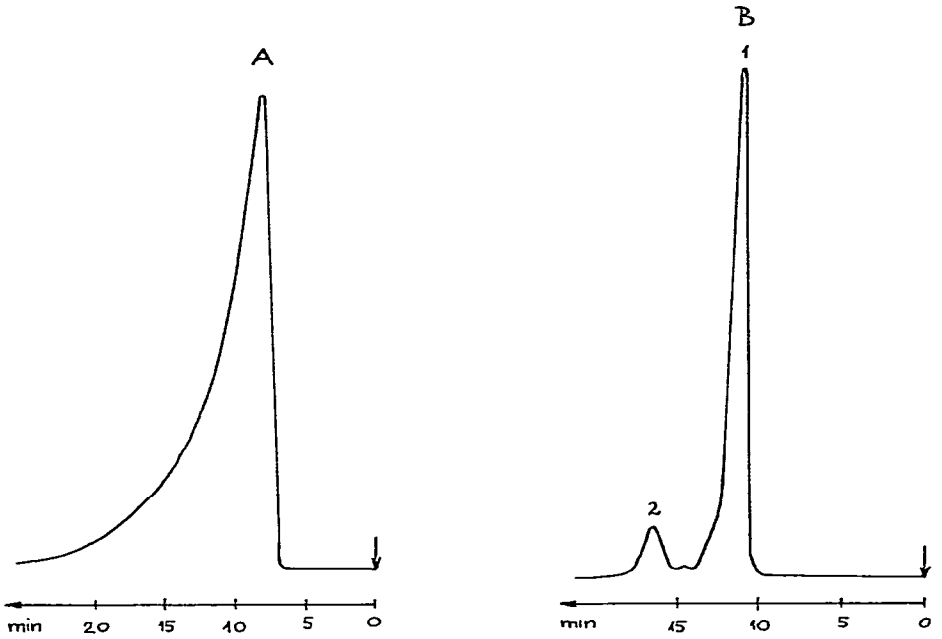

Fig. 1. Effect of salt concentration on the separation of bovine and porcine insulins. Column: Nucleosil 10 C_{18}, 250 × 4.6 mm I.D. Flow-rate: 1 ml/min. Detection : UV at 215 nm. Eluents: A, methanol–acetonitrile–buffered water, pH 2.2 (5:1:4); B, as A but buffer contained 0.1 *M* Na_2SO_4. Compounds: 1 = bovine insulin; 2 = porcine insulin.

Fig. 2 demonstrates the increased selectivity obtained by the change of the organic modifier in the eluent, for the separation of oxytocin raw product. The elution order was found to charge when acetonitrile was replaced with isopropanol in the eluent.

From the experimental data obtained on the solvent effect on the efficiency and selectivity of the isocratic separation of polypeptides, it can be concluded that on the one hand the use of mixed solvents as organic modifier in the eluent can increase the efficiency of the separation, whereas on the other the type of organic solvent has a significant effect upon the k' values of polypeptides, changing the selectivity of the separation.

Table I lists the capacity ratios measured for insulins using different organic solvents. Somewhat unexpectedly, the capacity ratios increased in the order isopropanol, tetrahydrofuran, acetonitrile, methanol.

Two types of eluent systems were found to be promising for the separation of polypeptides under isocratic conditions. The first system was a mixture of methanol, acetonitrile and buffered water containing 0.1 *M* sodium sulphate (see Experimental for eluents A–D) and this has proved to be suitable for the analysis of oxytocin,

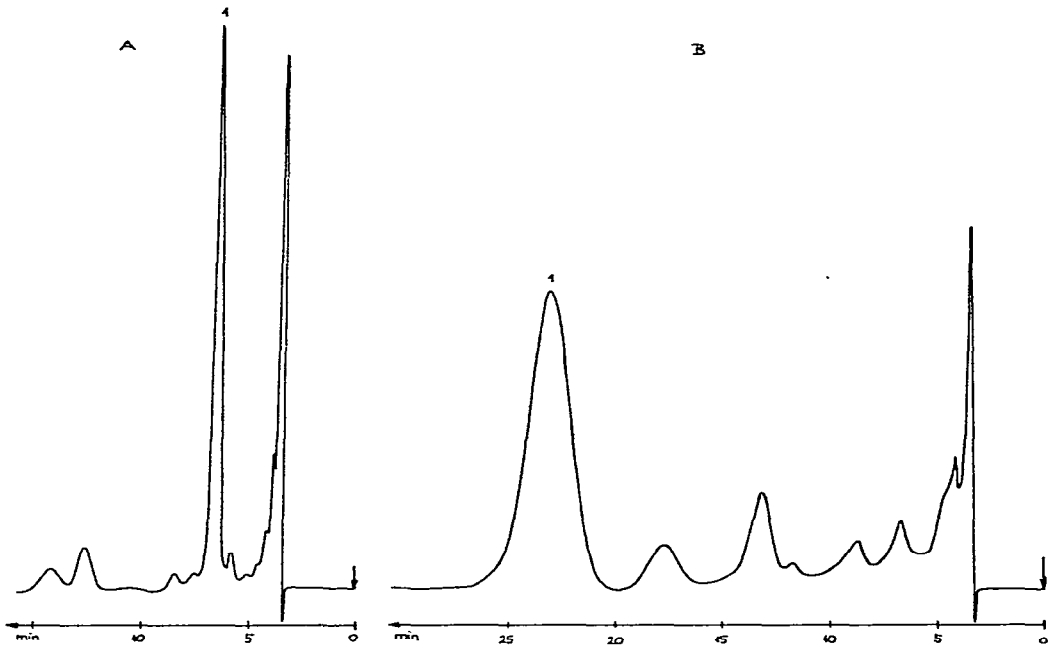

Fig. 2. Chromatograms of oxytocin raw product. A, Eluent A (see Experimental); B, Eluent E. Other conditions as in Fig. 1. Compounds: 1 = oxytocin; other peaks were not identified.

TABLE I

DEPENDENCE OF CAPACITY RATIOS, k', ON THE ORGANIC SOLVENT USED FOR PREPARATION OF ELUENT

Eluent: methanol–X–0.1 *M* $NaClO_4$, pH 2.2 (5:1:4), X_1 = Isopropanol; X_2 = tetrahydrofuran; X_3 = acetonitrile; X_4 = methanol.

	k'			
	X_1	X_2	X_3	X_4
Bovine insulin	0.17	1.01	2.69	4.23
Porcine insulin	0.18	1.95	4.10	9.10

$ACTH_{1-32}$ and aprotinine. The second system, comprising acetonitrile, isopropanol and buffered water containing 0.1 *M* sodium perchlorate (eluent F), can be used for the separation of insulins.

As regards, the pH of the eluent, all polypeptides except aprotinine can be separated at a pH of 2.2; aprotinine can be investigated at a pH 6.5.

O'Hare and Nice[12] described a very good gradient elution system for the separation of proteins and polypeptides. They used a Hypersil ODS column and an acetonitrile gradient at pH 2.1. On the basis of their retention data, Meek[13] calculated the retention coefficients of amino acid residues. He could then predict the retention

TABLE II

COMPARISON OF PREDICTED AND FOUND CAPACITY RATIOS OF ACTH DERIVATIVES

The predicted ratios were based on the retention times obtained by summing retention coefficients for each polypeptide. A retention time of 2.0 min was assumed for unretained compounds. See Experimental for eluents B and E.

	No. of residues	*k'*		
		Predicted pH 2.1	*Found*	
			Eluent B	*Eluent E*
$ACTH_{15-32}$	18	2.15	1.5	0
$ACTH_{1-32}$	32	13.8	7.5	4.7
$ACTH_{1-14}$	14	20.15	10.1	9.1
$ACTH_{1-24}$	24	26.25	13.9	17.7
$ACTH_{1-28}$	28	20.8	10.6	10.0

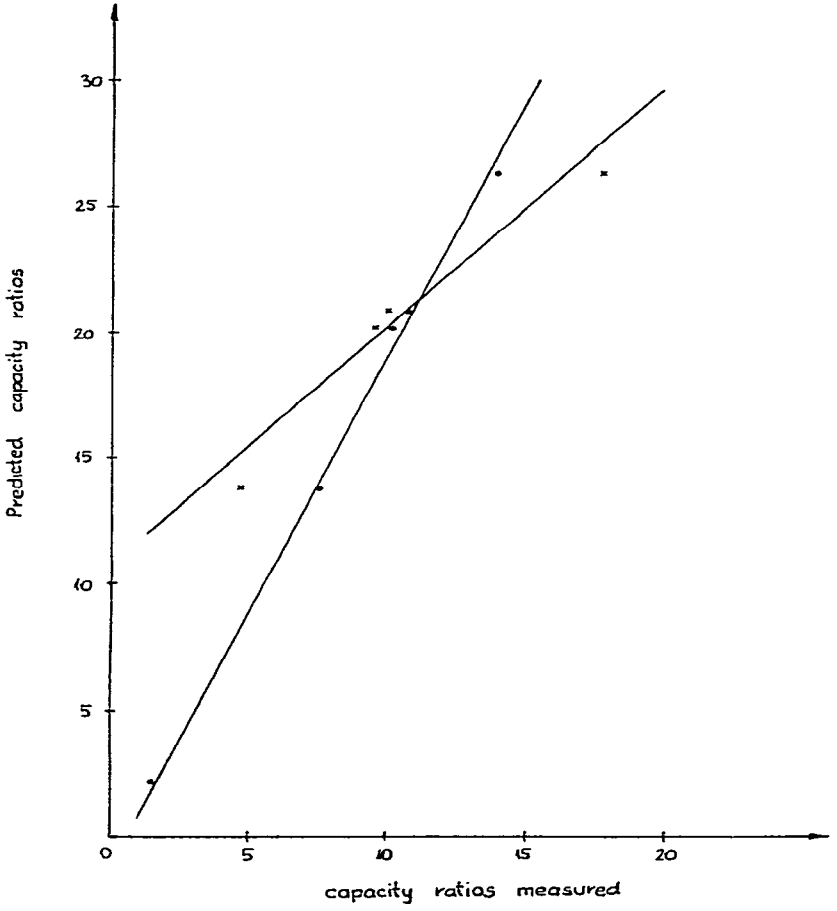

Fig. 3. Correlation of measured capacity ratios, *k'*, *vs.* capacity ratios predicted by summing retention coefficients for the amino acids and end groups present in ACTH derivatives. Eluents: ×, B; +, F.

times of different polypeptides. By summing the retention coefficients for each polypeptide; a good correlation was obtained between the predicted and found retention times.

We have examined the applicability of Meek's prediction method for isocratic elution with different eluents. Table II lists the predicted and found capacity factors obtained for ACTH derivatives. In Fig. 3 the predicted capacity factors are plotted against the capacity factors measured for ACTH derivatives using two different eluent systems. As is seen, a roughly linear relationship was obtained.

The predicted capacity ratios were calculated for the other polypeptides investigated and compared with the found values (Table III). The good correlation between estimated and found elution order indicates that the data calculated according to Meek can be transformed to isocratic conditions and different eluent systems. Thus the data in Table III can be used to select suitable chromatographic systems for the separation of polypeptides.

TABLE III

COMPARISON OF PREDICTED AND FOUND CAPACITY RATIOS FOR OXYTOCIN, APROTININE AND INSULINS

See Experimental for eluents A, D, E and F.

	k'					
	Predicted		*Found*			
	pH 2.1	*pH 7.4*	*Eluent A*	*D*	*E*	*F*
Oxytocin	13.3		1.31[a]		6.50	
Oxytocein			2.14		5.12	
Aprotinine		42.5		4.55		
Bovine Insulin	62.0					2.53
Porcine insulin	71.9					3.61
Bovine proinsulin	87.0					4.45
Porcine Proinsulin	95.9					12.90

Some applications of the method are now described. Fig. 4 shows the chromatogram of an oxytocin injection solution (10 I.U./ml). The relative standard deviation of the determination of oxytocin in formulations calculated for seven parallel runs was found to be 0.8% and a linear calibration curve was obtained within the concentration range 2–20 I.U./ml. When oxytocin concentrate (about 250 I.U./ml) was analysed the sample was diluted to about 10 I.U./ml.

Fig. 5 correlates the results obtained by HPLC and bioassay methods. Similar experiments were made by Krummen and Frei[18,19] who found that the regression values were within the range of variation expected for bioassay tests. Our data support their observation that more reliable results can be obtained by HPLC. The very good correlation between the results obtained by the two methods suggests the use of

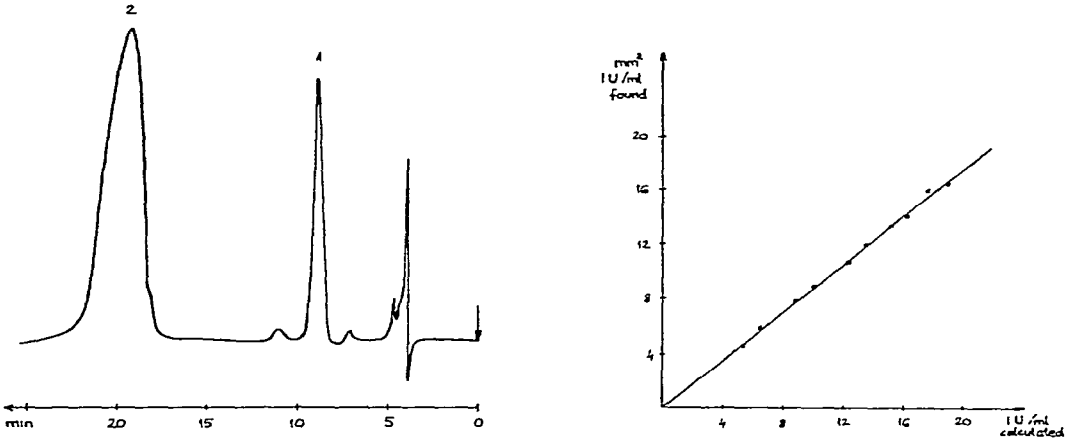

Fig. 4. Chromatogram of oxytocin injection solution (10 I.U./ml) and calibration curve for oxytocin. Eluent: A. Other conditions as in Fig. 2. Compounds: 1 = oxytocin; 2 = 4-trichlorobutanol-1.

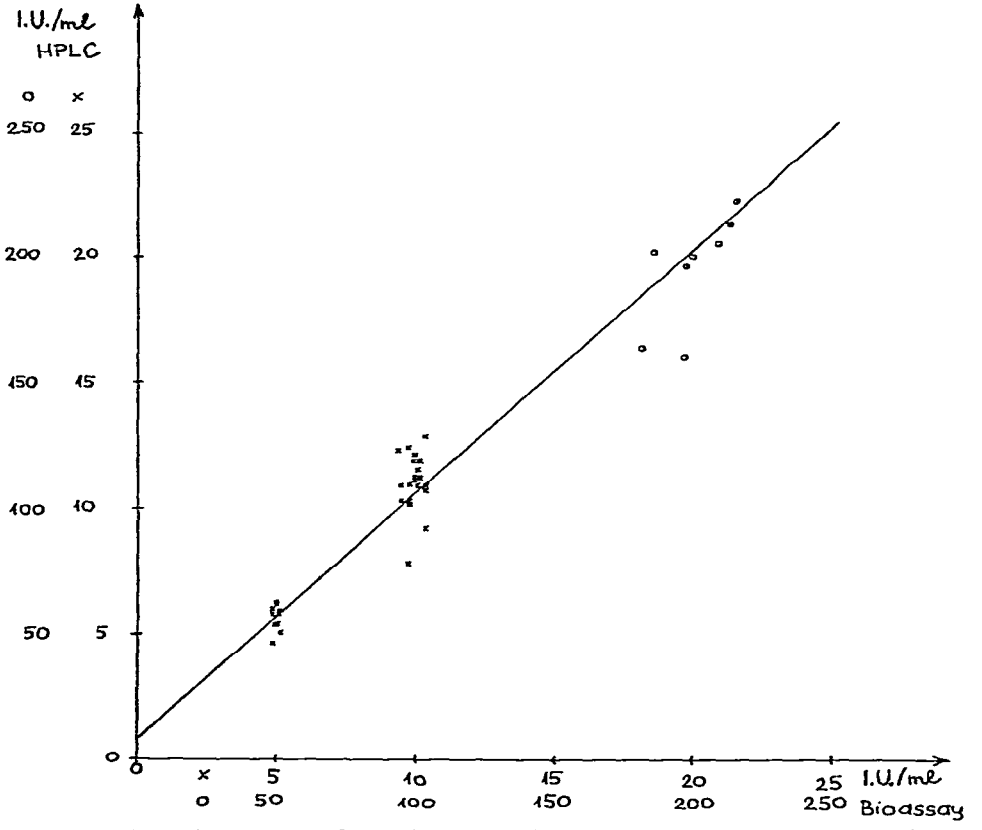

Fig. 5. Correlation between HPLC and bioassay results. Regression line (y = HPLC, x = bioassay): $y = 0.99\,x + 0.81$.

HPLC for the determination of oxytocin in pharmaceutical dosage forms and concentrates.

Fig. 6 shows the separation of aprotinine from Gordox® (Gedeon Richter) injection. In the case of aprotinine, satisfactory separation can be achieved only when the concentration of the organic modifier differs by less than $\pm 5\%$ compared to the optimal eluent system, because the separation efficiency is highly dependent on the concentration of the organic modifier.

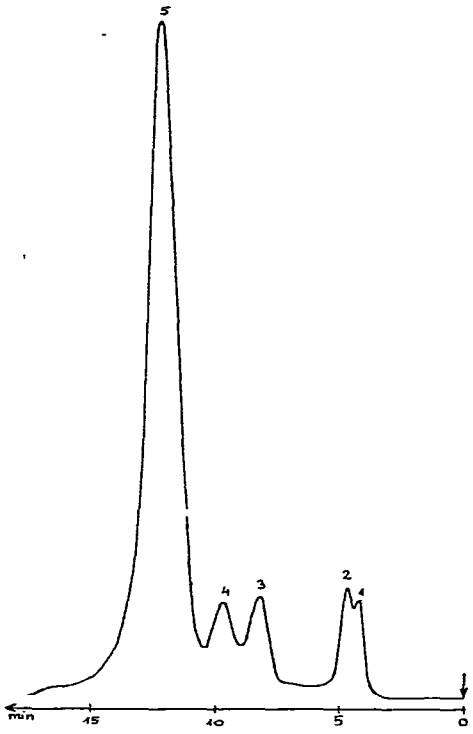

Fig. 6. Chromatograms of Gordox® injection. Eluent: D. Detection: 280 nm. Other conditions as in Fig. 1. Compounds: 1–4 = unknown; 5 = aprotinine.

The separation of insulins and related compounds by HPLC is a very important analytical task. We have reported our results elsewhere[20]. However, the optimization of the separation system was not considered. The importance of the choice of solvent can be demonstrated for insulin separation, Fig. 7 (see also Fig. 2 for oxytocin). The best separation was achieved by using eluent F. The separation of bovine and porcine insulins, desamidoinsulins and proinsulins is described elsewhere[20].

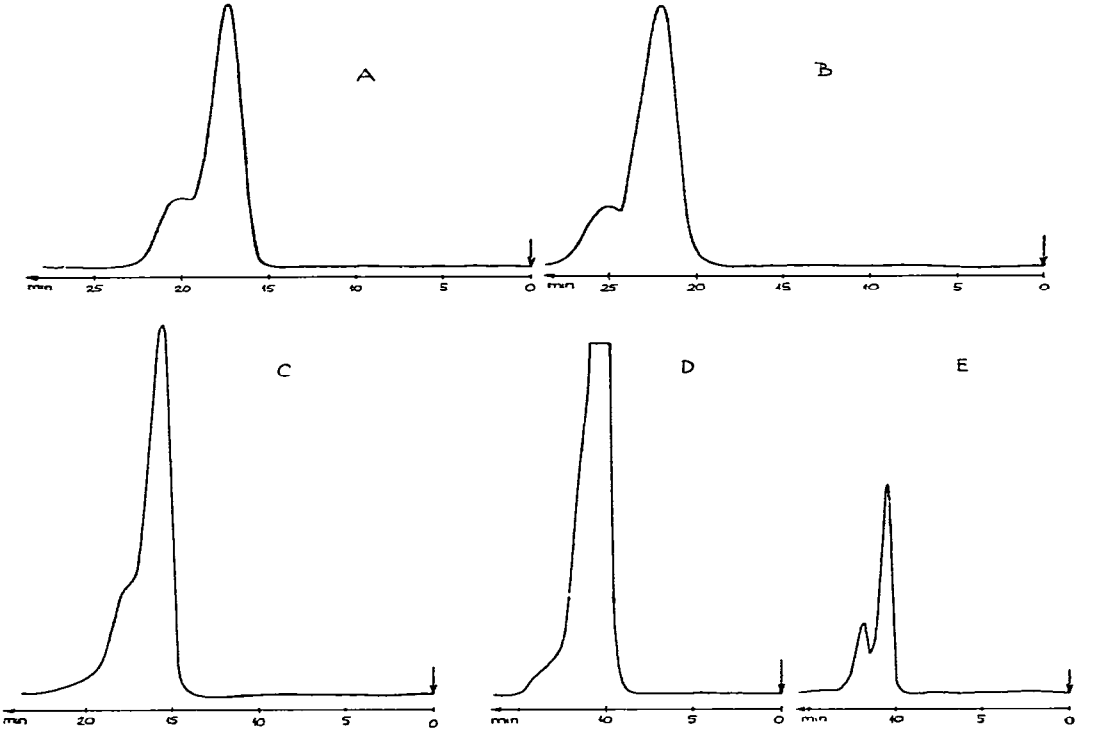

Fig. 7. Dependence of the separation of bovine insulin and desamidoinsulin on the eluent composition. Eluent: organic modifier aqueous 0.01 *M* phosphate buffer containing 0.1 *M* $NaClO_4$ (4:6). Organic modifier: A, methanol–isopropanol (4:2); B, methanol–isopropanol–acetonitrile (3:2:1); C, methanol–isopropanol–acetonitrile (2:2:2); D, methanol–isopropanol–acetonitrile (1:2:3); E, isopropanol–acetonitrile (2:4). Other conditions as in Fig. 1.

CONCLUSIONS

In the separation of polypeptides by hydrophobic chromatography it was found that both the type and concentration of the organic modifier as well as the salinity of the eluent have a significant influence on the selectivity and efficiency of the separation. The use of isopropanol as organic modifier in the eluent results in improved separations. It was also found that Meek's method of predicting retention times can be employed for isocratic elution with different eluent systems. The usefulness of this prediction method has been demonstrated for some polypeptides of biological interest.

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