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IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF INSULINS AND RELATED COMPOUNDS

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SUMMARY

A new method has been developed for the separation and determination of insulins and related polypeptides. Octadecylsilica was used as stationary phase and a mixture of water and organic solvents (methanol, acetonitrile and isopropanol) containing a relatively high amount of inorganic salt (sodium sulphate and sodium perchlorate) for controlling the degree of ionization as eluent. By this method the separation of bovine and porcine insulins, desamidoinsulins and proinsulins as well as that of intermediates can be achieved.

INTRODUCTION

Recently, there has been considerable interest in sensitive and specific analytical methods for monitoring the purity of insulins. In the past, disc electrophoresis[1–4] and gel chromatography[5–10] have been applied for this purpose. However, these methods are time-consuming and their accuracy, precision and efficiency are unsatisfactory. High-performance liquid chromatography (HPLC) has since been investigated for the separation of different insulins and related compounds, and great efforts have been made to find the most suitable system. Both reversed-phase partition chromatography[11–17] and reversed-phase ion-pair chromatography[17–20] have been studied in detail, however the separation of proinsulins, intermediates and degradation products of insulins has not been achieved.

In this paper we summarize our results in the field of insulin analysis. This work is a part of experiments aimed at the HPLC separation of large polypeptides. The explanation of the possible retention mechanism and discussion of the solvent and salt effects will be reported elsewhere[21].

EXPERIMENTAL

A Hewlett-Packard 1081/A liquid chromatograph equipped with a loop injector and a variable-wavelength UV-detector (Schoeffel Model 770) was used. The separations were performed on a Nucleosil 10 C_{18} column, 250 × 4.6 mm I.D. (Chrompack, Middelburg, The Netherlands).

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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). The insulin samples were from different sources; the bovine and porcine proinsulin were obtained from NOVO (Copenhagen, Denmark).

RESULTS AND DISCUSSION

Two different types of HPLC separation have previously been used for the analysis of insulins. In reversed-phase partition chromatography the ionization of the insulin molecule is controlled by the pH and salt concentration of the eluent and methanol or acetonitrile is used as organic modifier[11–16]. The compounds are sep-

TABLE I

CAPACITY RATIOS, k', FOR INSULINS AND RELATED COMPOUNDS

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–aqueous 0.01 *M* phosphate buffer, pH 2.2 (5:1:4), containing 0.1 *M* sodium sulphate; B, organic modifier–aqueous 0.01 *M* phosphate buffer, pH 2.2 (4:6), containing 0.1 *M* sodium perchlorate. The organic modifier contained 33.3% isopropanol in acetonitrile.

	k'	
	Eluent A	*Eluent B*
Bovine insulin	5.25	2.53
Bovine desamidoinsulin	6.00	2.90
Porcine insulin	7.75	3.61
Porcine desamidoinsulin	9.50	8.12
Bovine proinsulin	15.40	4.45
Porcine proinsulin	–	12.90

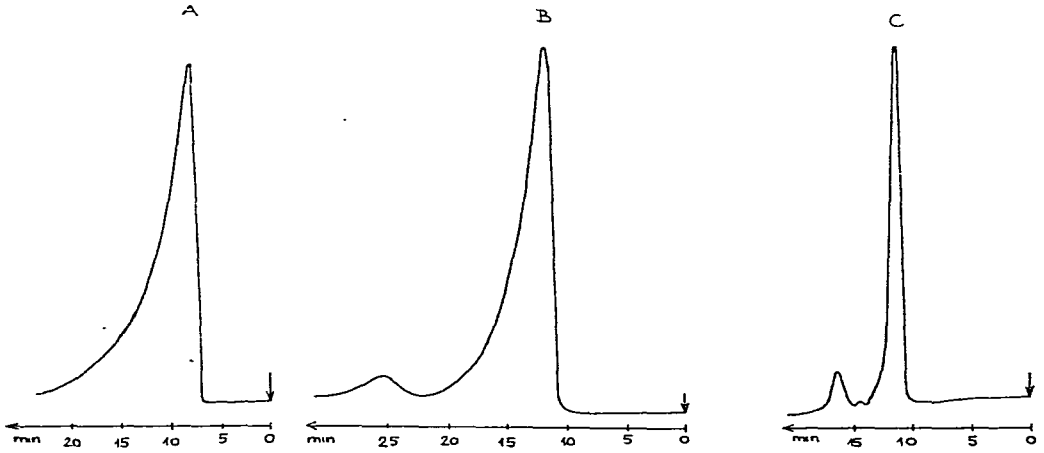

Fig. 1. Effect of the salt concentration on the separation of bovine and porcine insulins. Column: Nucleosil 10 C_{18}, 250 × 4.6 mm I.D. Flow-rate: 1 ml/min. Detection at 215 nm. Eluents: A, methanol–acetonitrile–buffered water, pH 2.2 (5:1:4); B, as A but buffer contained 0.01 *M* Na_2SO_4; C, as A but buffer contained 0.1 *M* Na_2SO_4.

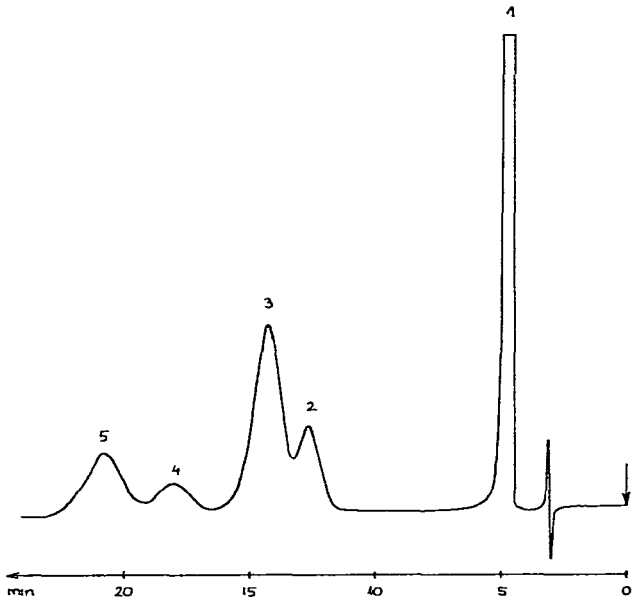

Fig. 2. Chromatogram of insulin injection decomposed by heat loading. Conditions as in Fig. 1, with eluent C. Compounds: 1 = ingredients; 2 = bovine insulin; 3 = bovine desamidoinsulin; 4 = porcine insulin; 5 = porcine desamidoinsulin.

arated on the basis of their different hydrophobicities. In reversed-phase ion-pair chromatography using anionic or cationic ion-pairing reagents[17–20], the compounds are separated as their ion pairs.

The first separation mode was chosen for our experiments, with octadecylsilica as stationary phase and a mixture of organic modifier and buffered water, pH 2.2, containing 0.1 *M* of an inorganic salt as eluent. The capacity ratios, k', measured for the compounds in two different eluent systems are collected in Table I.

Fig. 1 shows the separation of bovine and porcine insulins using an optimal mixture of methanol, acetonitrile and buffered water containing different amounts of sodium sulphate as eluent. The presence of the inorganic salt has a favourable effect

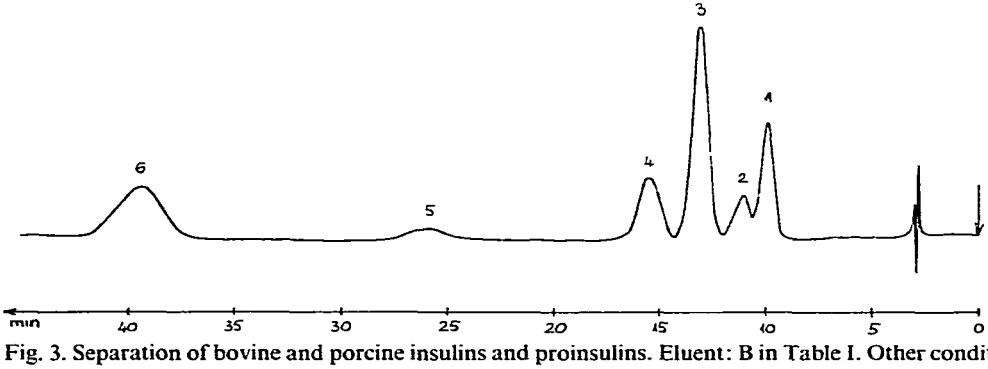

Fig. 3. Separation of bovine and porcine insulins and proinsulins. Eluent: B in Table I. Other conditions as in Fig. 1. Compounds: 1 = bovine insulin; 2 = bovine desamidoinsulin; 3 = porcine insulin; 4 = bovine proinsulin; 5 = porcine desamidoinsulin; 6 = porcine proinsulin.

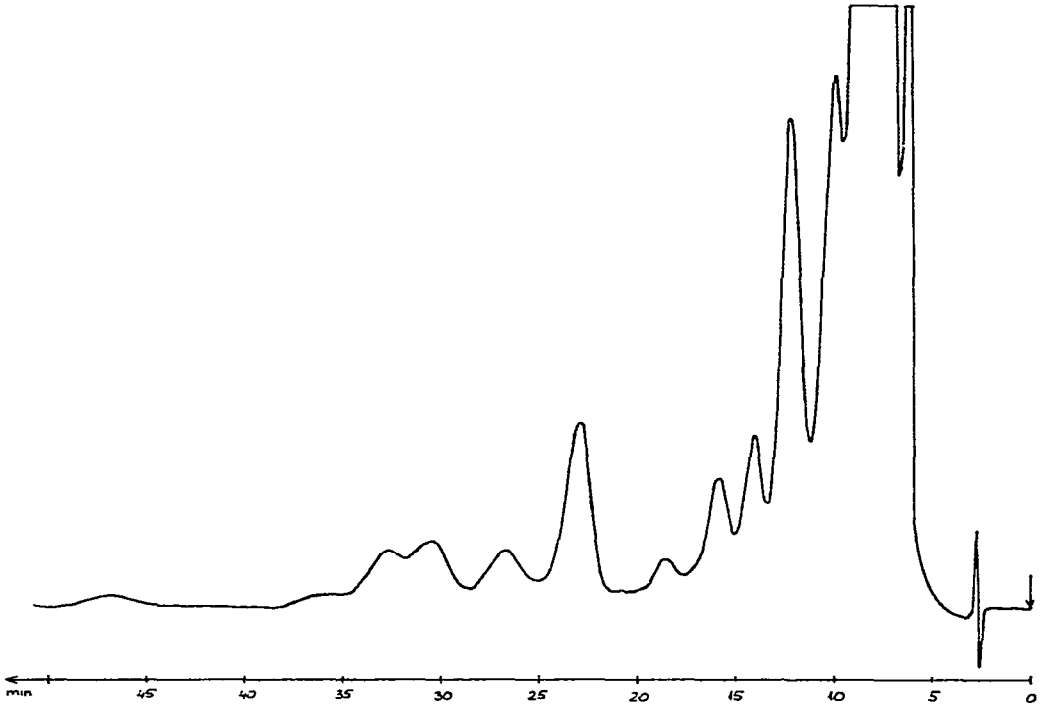

Fig. 4. Chromatogram of a commercial bovine insulin sample. Conditions: as in Fig. 3. Concentration: 500 μg per 20 μl.

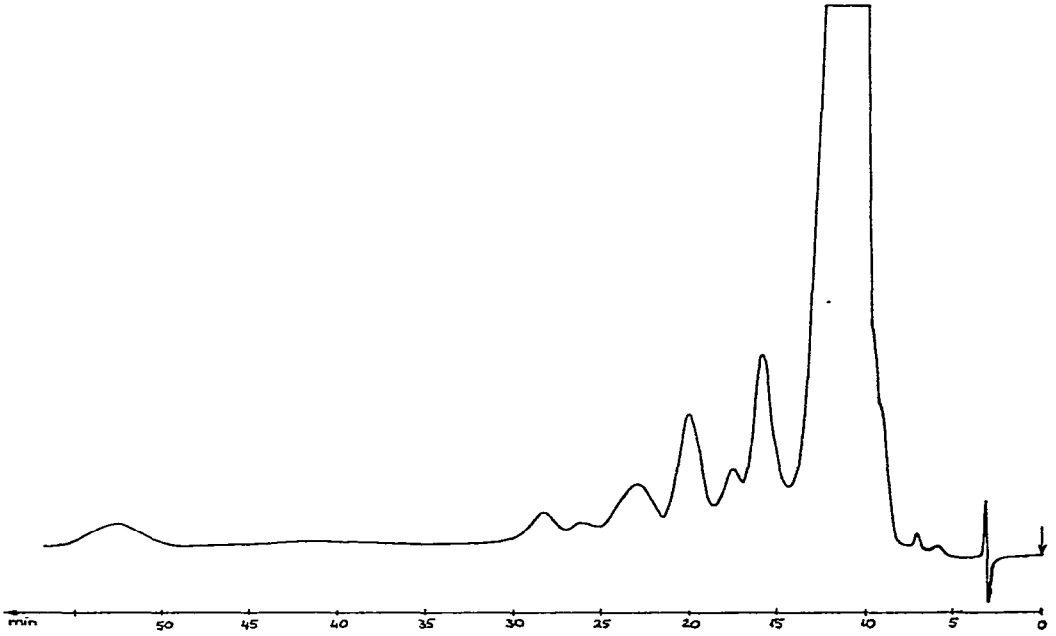

Fig. 5. Chromatogram of "so-called" proinsulin-free porcine insulin. Conditions as in Fig. 4.

on the separation efficiency, the peak shape being significantly improved. Fig. 2 demonstrates the applicability of this eluent to the investigation of a decomposed insulin injection (the decomposition was done by heat loading of the ampoules at 100°C for 5 h). A satisfactory separation of bovine and porcine insulins as well as their decomposition products (desamidoinsulins) was achieved.

When acetonitrile and isopropanol are used as organic modifier and the sodium sulphate is replaced with sodium perchlorate the efficiency of the separation can be increased further. This type of the eluent system facilitates the separation and identification of porcine and bovine insulins, desamidoinsulins and proinsulins, as shown in Fig. 3.

The number of theoretical plates for the compounds was calculated to be more than 5000, which suggests that this system may be more effective in the separation of impurities from insulins compared to previously published systems. This is illustrated in Figs. 4–6, when 500-μg insulin samples were injected. Fig. 4 shows the chromatogram of a commercial insulin satisfying the requirements of the British Pharmacopoeia 1980 Edition, while Fig. 5 illustrates the chromatogram of a "so-called" proinsulin-free purified insulin sample. For estimation of the quantities of impurities present in the samples 0.5 μg insulin were injected into the column, the resulting chromatogram can be seen in Fig. 6.

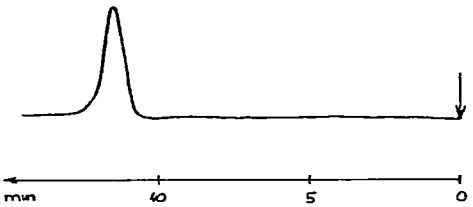

Fig. 6. Chromatogram of external standard solution. Concentration of insulin: 0.5 μg per 20 μl Conditions as in Fig. 3.

CONCLUSION

On the basis of the above results it can be concluded that the types and concentration of organic modifier used in the eluent are important in the separation of insulins and related compounds. A mixture of acetonitrile, isopropanol and buffered water, pH 2.2, containing 0.1 *M* sodium perchlorate has been found to be optimal for the separation and analysis of insulins.

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