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Analysis of Insulins by High-Performance Liquid Chromatography. II.[1] Separation of Various Species of Insulins[2]

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A reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the separation of bovine, porcine, ovine and equine insulins has been investigated. The complete chromatographic separation of these four insulins was achieved on a column of LiChrosorb RP-18 (5 μm) with a mixture of acetonitrile and 5 mM tartrate buffer (pH 3.0) containing sodium sulfate as the mobile phase at 40 °C. The capacity factors of the four insulins were greatly affected by the concentrations of acetonitrile and sodium sulfate in the mobile phase and by the column temperature.

Careful preparation of the mobile phase and a constant column temperature are essential for the reproducible and complete separation of insulins by this RP-HPLC method.

Keywords——HPLC; separation of insulins; bovine insulin; porcine insulin; ovine insulin; equine insulin; LiChrosorb RP-18

Recently, a reversed-phase high-performance liquid chromatographic (RP-HPLC) method has been widely used for the separation of proteins and peptides.[3] In the preceding paper,[1] an RP-HPLC method for the determination of insulin in preparations was described. Insulin was eluted on Nucleosil 5CN as a stationary phase with a mixture of acetonitrile–water–acetic acid (33 : 67 : 2) containing sodium octanesulfonate as the mobile phase.

RP-HPLC for the separation of various species of insulins and related compounds has been investigated in recent years.[4,5] Satisfactory separation of bovine, rabbit, human and porcine insulins by an RP-HPLC method using Zorbax TMS as the stationary phase was performed by Chance *et al.*[5a] Asakawa *et al.*[5b] reported the separation of bovine, porcine, ovine and equine insulins by an RP-HPLC method using Nucleosil CN as the stationary phase and a mixture of acetonitrile–phosphate buffer as the mobile phase, but broad peaks of those insulins resulted in unsatisfactory separation.

As it seems that the simultaneous separation of various insulins by RP-HPLC is useful for the identification and determination of insulin preparations, we have investigated and established a new RP-HPLC method for the separation of insulins from various sources using LiChrosorb RP-18 as the stationary phase, since this is cheaper than other similar stationary phases, with bovine, porcine, ovine and equine insulins as model insulins.

Experimental

Reagents and Materials——Bovine, porcine, ovine and equine insulins were purchased from Sigma Chemical Co. (U.S.A.). LiChrosorb RP-18 (5 μm) was obtained from E. Merck. Anhydrous sodium sulfate of special grade was obtained from Iwai Kagaku Co., Ltd. The other reagents (special grade) were from Wako Pure Chemicals, Ltd.

Apparatus and Chromatographic Conditions——A Hitachi model 635 high-performance liquid chromatograph equipped with a Hitachi wavelength-tunable effluent monitor was used. A stainless steel column (4ϕ × 250 mm) was packed with LiChrosorb RP-18 by a slurry-packing method. The column was operated at 40 ± 0.2 °C and at a flow rate of 0.8 ml/min. Detection was carried out at 280 nm (range: 0.04 O.D.). A mixture of acetonitrile and 5 mM

tartrate buffer (pH 3.0) (20:80) containing 0.57 M sodium sulfate was used as the mobile phase. The preparation of the mobile phase was performed at 20 °C in all cases. Columns were washed with a mixture of acetonitrile–0.2 N phosphoric acid (20 : 80) after use.

Sample Preparation——Each insulin (1 mg) was dissolved in 0.2 ml of 0.01 N hydrochloric acid.

Column Efficiencies—— The resolution between propylparaben and isopropylparaben on the column was more than 1.5 with a mixture of acetonitrile–water (2 : 3) as the mobile phase, corresponding to about 25000—30000 theoretical plates per meter.

Results and Discussion

Effect of Temperature

Figure 1 shows the capacity factors (k') of the four insulins at various temperatures. The change of temperature greatly affected the k' values of all the insulins. The k' values of all insulins increased with temperature from 20 to 50 °C. Linear relationships between log k' value and the temperature were obtained in the ranges of 20 to 40 °C and 40 to 50 °C. The elution order of the four insulins did not change at above 30 °C, and the elution order was as follows: bovine, ovine, equine and porcine insulins. The peaks of the four insulins were sharp at 20 °C, but the k' values were similar.

At 40—45 °C the separation of the mixture of the four insulins was complete, though the peaks were broadened slightly.

Thus, we employed 40 °C as the optimal temperature.

Effect of Acetonitrile Concentration

The k' values of four insulins at various concentrations of acetonitrile in the mobile phase at 40 °C are shown in Fig. 2. Change of the concentrations of acetonitrile in the mobile phase greatly affected the k' values of the four insulins. As the concentration of acetonitrile decreased from 21 to 19%, a significant increase in the k' values of all the insulins (about 15-fold) was noted. A linear relationship was obtained between log k' values and acetonitrile concentration in the range of 19 to 20.5%. Bovine, ovine, equine and procine insulins were eluted in that order. The peaks of the four insulins were sharp with 21% acetonitrile, but the k' values were similar.

In contrast, although the peaks of the four insulins were considerably broadened with 19% acetonitrile, their individual k' values differed from one another sufficiently to give mutual separation. The insulins were separated completely with 20% acetonitrile, though the

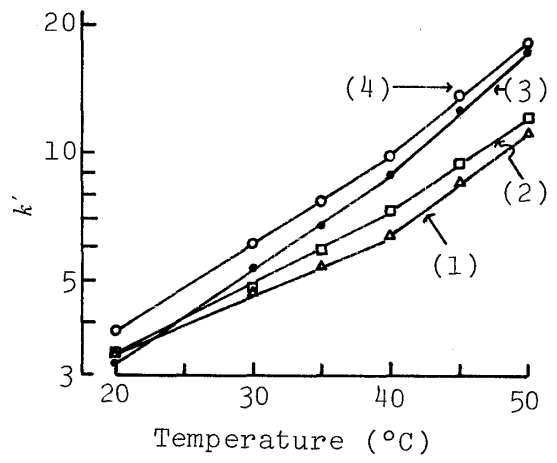

Fig. 1. Effect of the Column Temperature on the log k' Values of Various Insulins

Other conditions (except temperature) were the same as in Fig. 5.

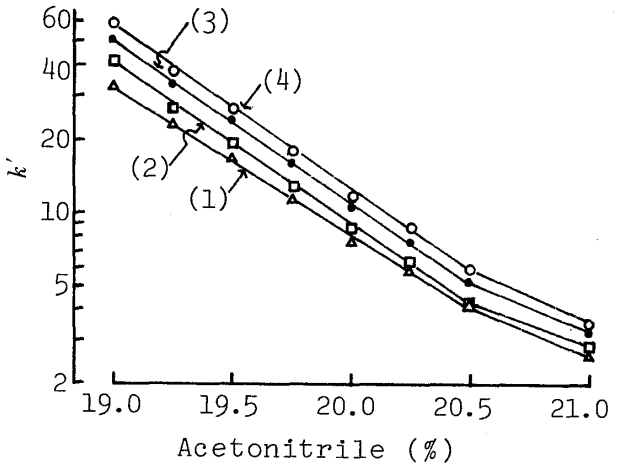

Fig. 2. Effect of the Acetonitrile Concentration on the log k' Values of Various Insulins

Other conditions (except acetonitrile concentrations in the mobile phase) were the same as in Fig. 5.

peaks were broadened slightly. Thus, we employed 20% as the optimal concentration of acetonitrile in the mobile phase.

Effect of the Concentration of Sodium Sulfate

The k' values of the four insulins at various concentrations of sodium sulfate in the mobile phase at 40 °C are shown in Fig. 3. Change of the concentration of sodium sulfate in the mobile phase greatly affected the k' values of insulins. As the concentration of sodium sulfate was decreased from 0.7 to 0.5 M, a significant increase in the k' values (about 15-fold) was noted. A linear relationship was obtained between $\log k'$ values and sodium sulfate concentration in the range of 0.5 to 0.6 M. Bovine, ovine, equine and porcine insulins were eluted in that order. The peaks of the four insulins were sharp at 0.7 M sodium sulfate, but the k' values were similar.

In contrast, although the peaks of the four insulins were considerably broadened at 0.5 M, their individual k' values differed from one another sufficiently to permit mutual separation. The separation of the four insulins was complete at 0.57 M, though the peaks were broadened slightly. Thus, 0.57 M sodium sulfate was employed as the optimal concentration in the mobile phase.

Effect of pH

The effect of the pH of 5 mM tartrate buffer in the mobile phase on the k' values of insulins at 40 °C is shown in Fig. 4. A linear relationship between the k' values and pH was obtained in the range of pH 2.8 to 5.0. Increase of pH resulted in a small decrease in the k' values of the insulins. The k' values of the four insulins were similar at pH above 4.0.

Thus, pH 3.0 was employed as the optimal pH of 5 mM tartrate buffer in the mobile phase.

When 50 mM tartrate buffer (pH 3.0) was used instead of 5 mM tartrate buffer in the mobile phase, the k' values of the insulins increased only 1.2-fold.

Separation of the Four Insulins

On the basis of the results described above, we established the optimal conditions for the separation of the four insulins. The optimal conditions were as follows: column temperature, 40 °C; mobile phase, acetonitrile–5 mM tartrate buffer (pH 3.0) (20 : 80) containing 0.57 M sodium sulfate. A typical chromatogram for the four insulins is shown in Fig. 5. The

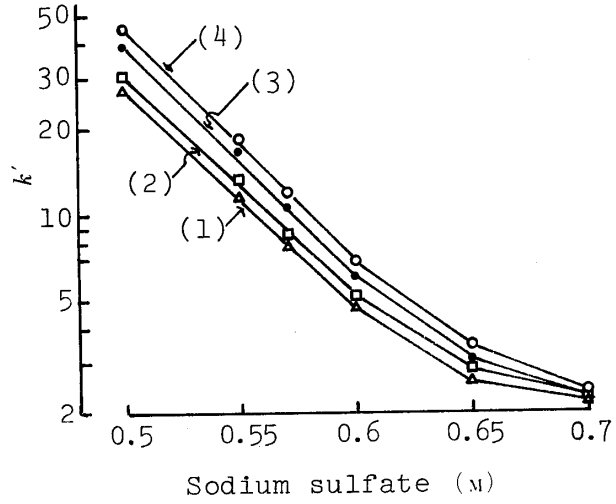

Fig. 3. Effect of the Concentration of Sodium Sulfate on the $\log k'$ Values of Various Insulins

Other conditions (except concentrations of sodium sulfate in the mobile phase) were the same as in Fig. 5.

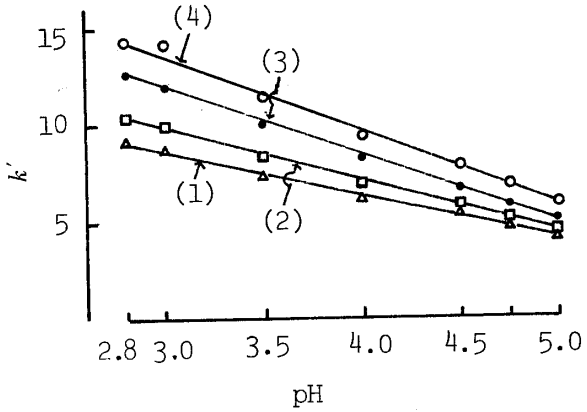

Fig. 4. Effect of pH of 5 mM Tartrate Buffer in the Mobile Phase on the k' Values of Various Insulins

Other conditions (except pH of tartrate buffer) were the same as in Fig. 5.

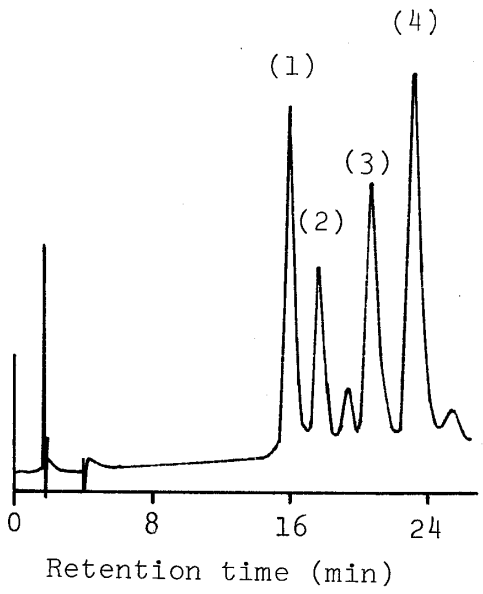

Fig. 5. Chromatogram of Various Insulins Obtained by the Established Method

Conditions of the established method: column, LiChrosorb RP-18 (4ϕ × 250 mm); column temperature, 40 °C; mobile phase, a mixture of acetonitrile–5 mM tartrate buffer (pH 3.0) (20 : 80) containing 0.57 M sodium sulfate; flow rate, 0.8 ml/min; detection, 280 nm (0.04 O.D.).

Compounds: (1) bovine insulin, (2) ovine insulin, (3) equine insulin, (4) porcine insulin. The load was *ca.* 10 μg of each insulin.

resolutions between bovine and ovine insulins, between ovine and equine insulins and between equine and porcine insulins were 1.5, 2.5 and 1.7, respectively. Therefore, the four insulins were separated completely. Mean k' values and their standard deviations for bovine, ovine, equine and porcine insulins were 7.2 ± 0.2 ($n=13$), 8.1 ± 0.2 ($n=13$), 9.7 ± 0.3 ($n=13$), 10.9 ± 0.4 ($n=13$), respectively. A satisfactory reproducibility of k' values was obtained under the established conditions. Because the concentrations of acetonitrile and sodium sulfate in the mobile phase and the column temperature greatly affected the k' values, careful preparation of the mobile phase and the maintenance of constant temperature were required to obtain reproducible separation of the insulins.

RP-HPLC separation methods for insulins published so far can be roughly divided into two groups based on isonization of insulins[4a,c,5a,b] and on ion-pair formation of insulins using ion-pair reagents.[4b,d,e] In the former case, a high salt concentration and low pH in the mobile phase were necessary for good peak shape and reproducibility.[4d] Insulins did not elute for 200 min on a LiChrosorb RP-18 column with a mobile phase of acetonitrile–5 mM tartrate buffer (pH 3.0) (20 : 80). Therefore, in our experiments, mobile phase containing a high concentration of sodium sulfate was employed. Terabe *et al.*[4b] reported an RP-HPLC separation on a Nucleosil $5C_{18}$ column with a mobile phase of acetonitrile–tartrate buffer containing sodium–1-butanesulfonate as an ion-pair reagent and sodium sulfate. However, this ion-pair reagent scarcely affected the peak shapes and k' values for bovine and porcine insulins on a LiChrosorb RP-18 column. Sodium dodecylsulfonate and sodium octylsulfonate also did not result in good separation of insulins. A successful separation of the four insulins was obtained by using a mobile phase without any ion-pair reagent. The components of the mobile phase used in our experiment are simple, cheap and offer good resolution.

Conclusions

(1) Increase of temperature results in a large increase in the k'values of insulins.

(2) Decrease of acetonitrile and sodium sulfate concentrations in the mobile phase results in a large increase in the k' values of insulins.

(3) The chromatographic separation of the four insulins was achieved with LiChrosorb RP-18 as the stationary phase and a mixture of acetonitrile–5 mM tartrate buffer (pH 3.0) (20 : 80) containing 0.57 M sodium sulfate as the mobile phase at 40 °C.

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References and Notes

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