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Analysis of Insulins by High-Performance Liquid Chromatography. III.[1] Determination of Insulins in Various Preparations[2]

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A reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of insulin in commercially available preparations has been developed. The HPLC method previously used for the separation of various insulins was subject to interference due to the preservatives in the preparations, so this HPLC method was modified. Insulin in preparations was determined without interference by the preservatives by means of HPLC method on a LiChrosorb RP-18 column using a mixture of acetonitrile and 5 mM tartrate buffer (pH 3.0) (27:73, v/v) containing 0.15 mM sodium sulfate as a mobile phase at 50 °C. The values obtained by the modified HPLC method were 95.0—108.8% of the labeled potency as the sum of potencies of both insulin and desamido-insulin, and the coefficients of variation were 1.03—3.14% ($n=3$).

Keywords——insulin HPLC; LiChrosorb RP-18; bovine insulin; porcine insulin; insulin preparation

Recently, high-performance liquid chromatographic (HPLC) method has been widely used for the separation of proteins and peptides.[3] However, the determination of insulin in commercial preparations by HPLC method has hardly been attempted. Compared with bioassay methods such as rabbit hypoglycemia assay, which is commonly used for insulin preparations, an HPLC method should be simple, accurate and precise, possibly permitting simultaneous determination of insulin and related compounds having insulin-like activity.

In a previous paper,[4] we reported the determination of insulin in preparations by an HPLC method using a Nucleosil 5CN column with a mixture of acetonitrile, water and acetic acid (33:67:2, v/v) containing sodium octanesulfonate as the mobile phase. However, bovine and porcine insulins gave the same retention time under the conditions used. We also reported a simultaneous separation of bovine, porcine, ovine and equine insulins by an HPLC method using a LiChrosorb RP-18 column with a mixture of acetonitrile and 5 mM tartrate buffer (pH 3.0) (20:80, v/v) containing 0.57 M sodium sulfate as the mobile phase.[1] However, because large amounts of preservatives in the preparations interfered with the determination of insulin, this HPLC method was also inapplicable to preparations.

In this paper, we describe an HPLC method for the determination of bovine and porcine insulins and related compounds in preparations without interference from the preservatives.

Experimental

Materials and Solutions——Bovine and porcine monocomponent insulins were purchased from Novo Industry. Their labeled potencies by bioassay were as follows: bovine insulin, 27.2 IU/mg; porcine insulin, 26.8 IU/mg. Desamido-insulin was prepared by the hydrolysis of insulin in 0.01 N hydrochloric acid at 37 °C for 4 weeks. Insulin preparations were obtained from 5 pharmaceutical companies. LiChrosorb RP-18 (5 μm) was obtained from E. Merck Co. The other reagents (special grade) were from Wako Pure Chemicals, Ltd.

Standard Stock Solution (SS): Bovine or porcine monocomponent insulin (10 mg) was dissolved in 2 ml of 0.01 N

hydrochloric acid. The solutions were stored at 0 °C and used within 1 month.

Internal Standard Solution (IS): *p*-Methylaminobenzoic acid (22 mg) was dissolved in 0.001 N hydrochloric acid and made up to 1000 ml.

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 (250 mm × 4 mm i.d.) was packed with LiChrosorb RP-18 by a slurry-packing method. The column temperature was maintained at 50 °C, and the flow rate was 0.8 ml/min. Detection was carried out at 280 nm. A mixture of acetonitrile and 5 mM tartrate buffer (pH 3.0) (27 : 73, v/v) containing 0.15 M sodium sulfate was used as the mobile phase. The preparation of the mobile phase was performed at 20 °C. The column was washed with a mixture of acetonitrile and 0.1 M phosphoric acid (27 : 73, v/v) after use.

Assay by HPLC Method——An insulin preparation corresponding to 10—20 IU of insulin was accurately measured and dissolved in 10 μl of 1 N hydrochloric acid, then 100 μl of IS was added. If necessary, an appropriate volume of 0.001 N hydrochloric acid was added to make 610 μl total volume (sample solution). For the standard solution, bovine insulin SS (100 μl), porcine insulin SS (50 μl) and 100 μl of IS were mixed, Next, an appropriate volume of 0.001 N hydrochloric acid was added to make 610 μl total volume. Aliquots (20 μl) of standard solution and sample solution were alternately injected onto the HPLC column. The peak heights of insulins and internal standard were measured. The peak height ratios of insulins with respect to the internal standard were calculated.

Results and Discussion

HPLC Conditions for Separation of Insulins, Desamido-Insulins and Preservatives

Insulin preparations usually contain phenol, cresol or methyl *p*-hydroxybenzoate as preservatives, and insulin related compounds such as desamido-insulin are also contained in some preparations. Because the HPLC method described in our previous paper[1] for the separation of various insulins was subject to interference by the preservatives and could not successfully separate insulin and its analog, the HPLC conditions were modified in this work. When a mixture of acetonitrile and 5 mM tartrate buffer (pH 3.0) (27 : 73, v/v) containing 0.15 M sodium sulfate was used as the mobile phase, bovine and porcine insulins and their analogs were separated satisfactorily at above 30 °C. However, the retention times of

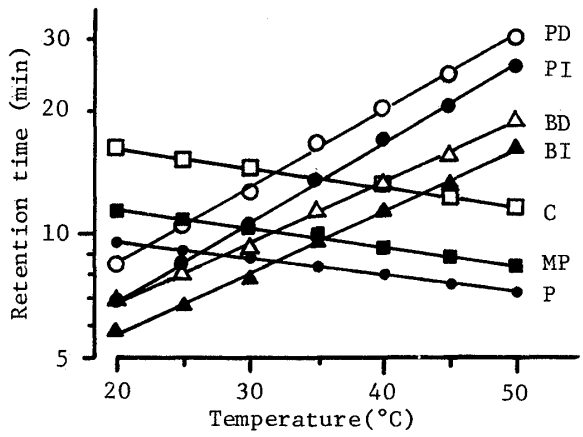

Fig. 1. Effect of the Column Temperature on the Retention Times of Various Insulins and Preservatives

Compounds: P, phenol; MP, methyl *p*-hydroxybenzoate; C, *m*-cresol; BI, bovine insulin; BD, bovine desamido-insulin; PI, porcine insulin; PD, porcine desamido-insulin.

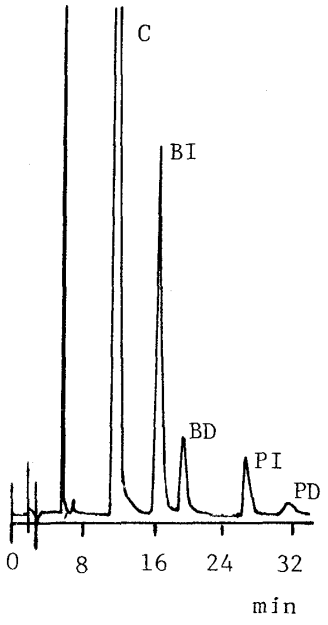

Fig. 2. Chromatogram of Insulin Preparation O

Compounds were the same as in Fig. 1.

TABLE I. Assay Results for Bovine and Porcine Insulins in Preparations as Determined by HPLC

Sample	Labeled potency (IU/ml)	Found (IU/ml) ($n=3$)					
		BI	BD	PI	PD	BI+PI	Total
A	40	27.0	—	12.9	—	39.9	39.9
B	40	26.7	—	12.5	—	39.2	39.2
C	40	26.8	—	12.7	—	39.5	39.5
D	40	27.3	1.0	12.9	—	40.2	41.2
E	40	26.9	1.0	13.3	—	40.2	41.2
F	40	28.9	1.0	13.6	—	42.5	43.5
G	40	19.6	2.8	13.9	1.7	33.5	38.0
H	40	34.5	1.7	3.5	—	38.0	39.7
I	40	34.5	1.8	2.5	—	37.0	38.8
J	100	86.1	4.6	6.3	—	92.4	97.0
K	40	32.5	—	7.8	—	40.3	40.3
L	40	18.1	8.9	7.6	3.5	25.7	38.1
M	20	13.0	3.8	2.4	0.6	15.4	19.8
N	40	24.2	8.0	4.5	1.5	28.7	38.2
O	40	26.2	5.8	5.6	1.3	31.8	38.9

BI, bovine insulin; BD, bovine desamido-insulin; PI, porcine insulin; PD, porcine desamido-insulin; total, BI+BD+PI+PD.

preservatives were similar to those of insulins and their analogs in the temperature range of 30 to 45 °C (Fig. 1). At 50 °C, bovine and porcine insulins, their desamido-insulins and preservatives could be separated completely. Under the above conditions, the working curves for bovine and porcine insulins were linear from 0 to 20.5 IU/ml and from 0 to 24 IU/ml, respectively, and passed through the origin.

Application to Insulin Preparations

The modified HPLC method described above was applied to the commercial preparations. Because the biological activity[5a] and the absorption coefficient at 280 nm[5b] of desamido-insulin are similar to those of insulin, bovine and porcine monocomponent insulins were used as reference standards for the determination of bovine and porcine desamido-insulins, respectively. A typical chromatogram of one preparation (sample O) is shown in Fig. 2. Mutual separation of cresol, bovine and porcine insulins and their desamido-insulins was complete. The assay results for 15 kinds of commercial preparations are shown in Table I. The sums of the potencies of insulins and desamido-insulins were 38.0—43.5 IU/ml (labeled potency: 40 IU/ml), 19.8 IU/ml (labeled potency: 20 IU/ml) and 97.0 IU/ml (labeled potency: 100 IU/ml). The coefficients of variation were 1.03—3.14% ($n=3$). Thus, the sums of potencies of insulins and desamido-insulin obtained by the modified HPLC method were similar to the labeled potencies of the preparations, and were 95.0—108.8% of the labeled potencies.

The determination of bovine and porcine insulins and their desamido-insulins in preparations can be accomplished without interference by the preservatives by means of the HPLC method described in this paper.

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References and Notes

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