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Note

High-performance liquid chromatographic determination of bovine and porcine insulins in a commercial injection*

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In a previous paper[1], we presented a reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of lysozyme chloride in some commercial antiphlogistics. The method, employing a short 50-mm column and an elution gradient from aqueous acidic phosphate buffer (solvent A) to a ternary mixture (solvent B) of 2-propanol, ethylene glycol and solvent A, afforded a high yield (97%) of lysozyme chloride from the column.

In the present work, we applied this high-yield method on a commercially available column (Shim-pack FLC-ODS) to afford a high and fairly constant recovery and satisfactory separation of bovine and porcine insulins in an injection.

Methods of simultaneous determination of bovine and porcine insulins using an isocratic elution technique have also been reported recently by Lloyd and Corran[2], Lloyd[3] and Ohta *et al.*[4] We report here a gradient elution technique, which gives convenient and reproducible results.

EXPERIMENTAL

Materials and reagents

Crystalline bovine and porcine pancreatic insulins (Novo Research Institute, Denmark) and a biphasic insulin injection (Novo Yakuhin K.K., Tokyo) were all commercial products. 2-Propanol (HPLC grade), ethylene glycol (amino acid analysis grade) and other special grade reagents (85% orthophosphoric acid, $NaH_2PO_4 \cdot 2H_2O$) were purchased from Wako, Osaka, Japan.

Apparatus and chromatographic conditions

A Shimadzu Model LC-2 high-performance liquid chromatograph equipped with a gradient elution unit (Model GRE-2) and a UV spectrophotometric detector (Model SPD-2A) was used. The commercial column used (Shim-pack FLC-ODS, 50 × 4.6 mm I.D., 3 μm, Shimadzu), exhibited 4000 theoretical plates for acenaphthene with acetonitrile–water (3:2). The column temperature was maintained at 37°C with a column oven (Model CTO-2A), and the flow-rate was 0.5 ml/min. Insulin analysis

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was carried out by use of a complex-linear gradient from 100% of solvent A (0.1 *M* NaH_2PO_4, adjusted to pH 2.0 by adding 6 ml of 85% (w/w) orthophosphoric acid to 1 l of 0.1 *M* NaH_2PO_4 solution) to 55% solvent A–45% solvent B (2-propanol–ethylene glycol–1 *M* NaH_2PO_4, 5:3:2 by volume ratio; adjusted to pH 2.5 by adding 85% orthophosphoric acid). The following gradient elution curve was employed: the volume percent of solvent B was increased linearly from 0% to 30% in 3 min (at 10%/min), and from 30 to 45% in 30 min (at 0.5%/min) and then was returned to 0% instantaneously; 15 min were required for recycling to the initial conditions and 48 min for one cycle of determination. Detection of protein was carried out at 210 nm (range 0.64 a.u.f.s.).

Sample preparation

Standard insulins (*ca.* 10 mg) were dissolved in 10 ml of solvent A, and stored in a freezer (at −20°C). Diluted injection samples, 1 ml of a biphasic insulin injection in 9 ml of solvent A, may also be stored at −20°C.

Determination process

Simultaneous determination of porcine and bovine insulins in an injection sample (diluted as above) was carried out as follows. A 4-μl volume of standard solution, which contained 1.04 mg/ml of bovine insulin and 0.64 mg/ml of porcine insulin, was injected into the HPLC system. Peak area values (μV sec) corresponding to porcine and bovine insulin peaks were incorporated into a data processor (Model C-R1A). The correlation factors between the peak areas and the amount of insulins injected were also calculated. Determination was carried out by the absolute calibration curve method (external standard method). Then 50 μl of diluted sample was injected into the HPLC system, and the peak areas of the sample were processed for quantitative determination. Coefficient of variation (C.V.) values were also calculated with a data processor (Model C-R2AX) by a BASIC program.

Recovery of bovine and porcine insulins from an injection

Amounts of 0.975 mg and 0.596 mg of bovine and porcine insulin, respectively, were added to 1 ml of undiluted biphasic insulin injection, and diluted with Solvent A to 10 ml. Determinations were carried out as described above. Recovery values were obtained from the difference of insulin analyses in the biphasic insulin injection with and without added standard insulins.

RESULTS AND DISCUSSION

A chromatogram of standard bovine and porcine insulins obtained by the method described as in Experimental is shown in Fig. 1. The amount of bovine and porcine insulins injected and the observed peak area were linearly correlated, as shown in Fig. 2. The lower detection limit was 50 ng (S/N = 2) for both insulins. The repeatability of the method (Table I) was satisfactory for eight repeated cycles with C.V. values as low as *ca.* 2%.

We applied this method to the simultaneous determination of bovine and porcine insulins in an injection. A typical chromatogram of an injection sample is shown in Fig. 3, and the determination process is shown in Fig. 4. The quantitative values

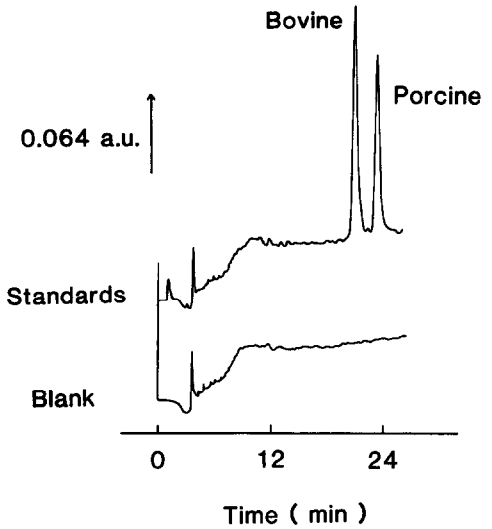

Fig. 1. A chromatogram of standard bovine and porcine insulins. Standard bovine and porcine insulin mixture, which contained 2.81 and 1.86 μg of bovine and porcine insulin, respectively, was injected into the HPLC system.

are listed in Table II. The C.V. values of the sample were somewhat higher (*ca.* 4%). However, lower C.V. values may be obtained by scaling up the sample dilution step.

From the results in Table II, we determined the recoveries of bovine and porcine insulins from an injection sample. As shown in Table III, the recoveries of this

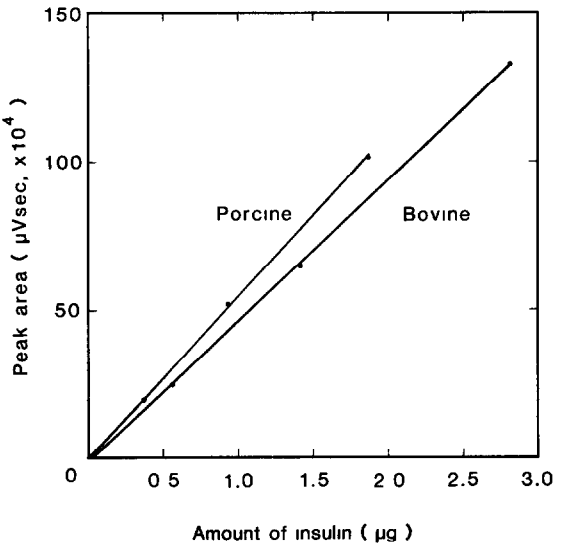

Fig. 2. Calibration curves for bovine and porcine insulins.

TABLE I

REPEATABILITY OF INSULIN DETERMINATION

Loading: bovine insulin, 2.81 μg; porcine insulin, 1.86 μg. Peak areas were measured with a data processor (Chromatopac C-R2AX, Shimadzu Corporation).

Run number	*Peak area (μV sec)*	
	Bovine	*Porcine*
1	1,220,584	921,678
2	1,254,668	926,854
3	1,228,640	939,065
4	1,189,196	953,881
5	1,260,734	920,495
6	1,227,739	946,330
7	1,215,369	930,992
8	1,219,426	894,548
Average	1,227,045	929,605
S.D.*	22,574	18,285
C.V.	1.84%	1.97%

* Standard deviation (unbiased form).

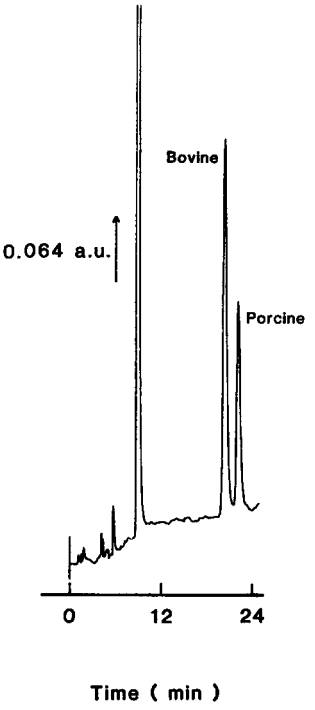

Fig. 3. A chromatogram of an injection sample. A major peak eluted near 10 min was identified as methyl *p*-hydroxybenzoate (preservative). 50 μl of diluted injection sample was analysed.

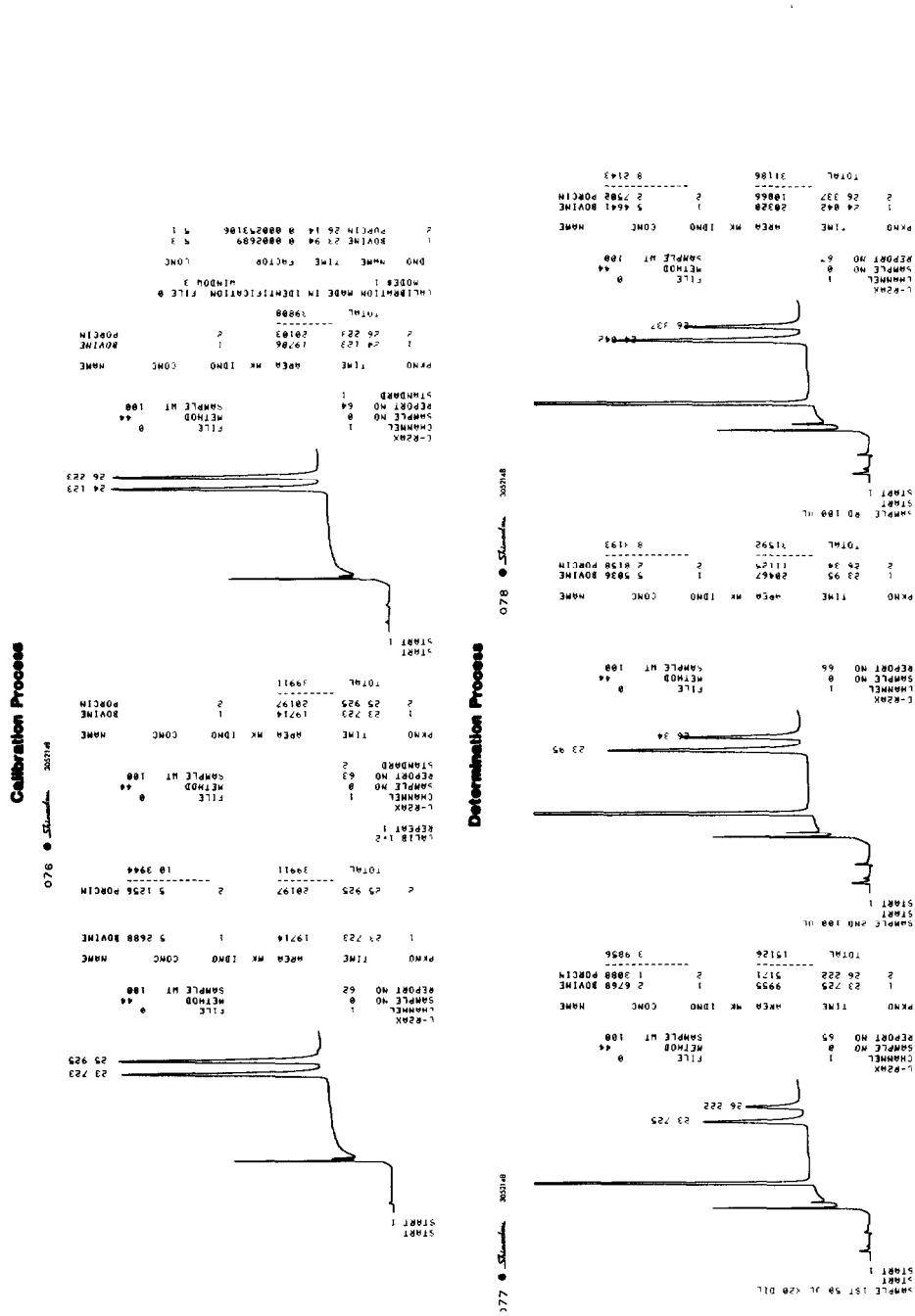

Fig. 4. A typical example of the determination process. A 20-fold diluted sample was used, and 4 h were sufficient for the quantitative analysis of a biphasic injection. In this case, an LC-4A pump and a data processor C-R2AX were used. The sample volumes injected in the second row from left to right were 50, 100, and 100 μl, respectively. Other conditions were as in Fig. 1.

TABLE II

DETERMINATION OF BOVINE AND PORCINE INSULINS IN AN INJECTION

A 1-ml volume of the injection solution was diluted with 9 ml of solvent A; 50 μl of the diluted sample was loaded.

Sample number	*Bovine insulin (mg/ml)*	*Porcine insulin (mg/ml)*
1	1.017	0.5326
2	0.965	0.4895
3	0.997	0.5184
4	0.958	0.4967
Average	0.984	0.5084
S.D.	0.0279	0.0193
C.V.	2.83%	3.80%

TABLE III

RECOVERY OF STANDARD BOVINE AND PORCINE INSULINS FROM AN INJECTION

Contents of bovine and porcine insulins used were 0.984 mg/ml and 0.5084 mg/ml, respectively. 0.975 mg standard bovine insulin and 0.596 mg standard porcine insulin were added.

Test No.	*Bovine insulin (found)*	*Porcine insulin (found)*
1	0.9997	0.6369
2	0.9602	0.5665
3	1.0046	0.6379
Average	0.9882 (101.3%)	0.6138 (103.0%)
S.D.	0.0243	0.0409
C.V.	2.5%	6.7%

method were 101% for bovine insulin and 103% for porcine insulin. Thus, this method was shown to be applicable to simultaneous determination of bovine and porcine insulins in a biphasic injection.

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