Determinetermination of Purity and Identification of Animal Sources of Insulin in Various Insulin Preparations

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Abstract—Prolonged administration of insulin leads to the formation of insulin-binding antibodies due to contaminant peptides and the animal source of the insulin. It follows that quantitation and identification of these factors are of significant importance in pharmaceutical insulin preparations. The assay and test procedures stipulated in the current pharmacopoeia of various countries, nevertheless, cannot determine either of these effects. In the present study, the content of impurities in insulin preparations was measured by polyacrylamide gel disc electrophoresis and the animal source of insulin identified by amino acid analysis. Assays of 17 commercial insulin preparations by these techniques revealed diversity in purity and animal sources of insulin. The present results suggest potential usefulness of these assay methods and advisability of their adoption not only by the manufacturers but also by the official pharmacopoeia as well.

Since insulin was first used therapeutically by Frederick G. Banting in the management of diabetes in humans (1), parenteral preparations of insulin have become indispensable in the treatment of diabetes mellitus. With progress in biological and chemical sciences, considerable improvement has been made in pharmaceutical methods and various forms with different lengths of activity are now available. Mirsky and Kawamura (2) demonstrated by electrophoretic analysis, however, that related peptide contaminants are present in these pharmaceutical preparations of insulin. In recent years, Schlichtkrull et al. (3, 4) demonstrated the relation of peptide contaminants in the production of insulin-binding antibody and proposed that ion-exchange chromatography should replace the conventional recrystallization method for purification of insulin. Thus, new monocomponent insulin preparations have been developed. Their contention was that the high antigenicity of insulin despite its low molecular weight is attributable to the presence of impurities and this has given new impetus to development of more sensitive tests to determine the purity of insulin in pharmaceutical preparations. The tests for purity currently described in the British Pharmacopoeia, United States Pharmacopoeia, and Japanese Pharmacopoeia are based solely on the assay for total proteinous nitrogen content of preparations. This is an extremely gross means of determining the purity of insulin, especially when one considers that some of the impurities may be polypeptides. Therefore, a more sensitive test with which polypeptide impurities could be detected, is required. The present study was such...
Because antigenicity of insulin preparations has acquired considerable importance in clinical practice, it is essential to identify the species of the animal from which the insulin has been isolated. Bovine insulin is more antigenic to man than is porcine insulin because of the greater difference in amino acid composition from that of human insulin. The above three pharmacopoeias provide no method for identification of the animal species from which the insulin was obtained. Thus it is left to the distribution, with the exception of British Pharmacopoeia by which the Pharmaceutical company is required to identify the animal species on the label of individual containers. Considering the difference in amino acid composition of peptide chains between bovine and porcine insulin, we devised a method for identification of insulin from different animal sources, using an amino acid autoanalyzer.

MATERIALS AND METHODS

**Materials:** Insulin preparations—Insulin preparations used were 17 commercial preparations of insulin available in Japan, i.e. crystalline insulin zinc suspension (A-G), isophane insulin (H-J), biphasic insulin (K), amorphous insulin zinc suspension (L-M) and soluble insulin preparations (N-Q). Other reagents were all special grade commercial products.

**Method for Purity Test:** Porcine monocomponent insulin (Novo Industry, Copenhagen), was used as the standard and prepared in solutions in 8 M urea to concentrations of 0.25, 0.5, 1.0, 5.0, 10.0, 100.0, and 1000 μg/100 μl. Using the procedure previously described (5), these standard solutions and test samples were applied to 1,000 μl of 7.5% polyacrylamide gel for separation and 150 μl of 2.5% polyacrylamide gel for concentration in glass tubes (5 x 65 mm) to run disc gel electrophoresis using an electrical charge of 1.5 mA/tube for the first 10 min and increasing to 3 mA/tube until just before disappearance of the marker. The disc gel was stained with Amido Black (Wako, Tokyo) dissolved in acetic acid and decolorized with 3% acetic acid. Seventeen commercial preparations of insulin (40 U/ml) currently available in Japan were tested. Fifty microliters of the test sample (equivalent to approx. 80 μg of insulin) were combined with an equal volume of 8 M urea, and electrophoretic mobility of the mixture was then analyzed on polyacrylamide gel to determine the percentage content of impurities in the sample, by comparing the bands representing them (i.e., those other than the central insulin band) with the intensity of color obtained by electrophoresis of the standard preparation. Serial concentrations of the standard solution were adjusted to the concentration of impurities when the test samples showed a high content of contaminants. The test has to be repeated if no gradation is observed in the intensity of staining of the gels prepared with standard solutions. The sensitivity of the assay for insulin was 0.25 μg.

**Method for Identification of Species of Animal Sources:** The amino acid composition of insulin preparations was analyzed by the following procedure: to 3 mg of the test sample, 2 ml of 6 N hydrochloric acid was added. The resulting solution was placed in a glass ampule which was then sealed under reduced pressure and heated for 24 hr at 110±2°C.
When cooled, it was opened and the content was evaporated to dryness under reduced pressure. The residue was dissolved in 2.5 ml of citrate buffer, pH 2.2, and the amino acid concentrations in the solution were determined with a Hitachi KLA-5 amino acid autoanalyzer, using a Hitachi Custom No. 2613 chromatographic column (9 x 550 mm) for acid and neutral amino acids and Hitachi Custom No. 2611 column (9 x 100 mm) for basic amino acids. The data thereby obtained were processed in a Hitachi chromato-processer, Model 834.

In case of insulin suspensions, 2 ml of the sample were centrifuged at 3000 rpm for 10 min and, after discarding the supernatant, 2 ml of 6 N hydrochloric acid was added to the sediment to allow for amino acid analysis, by the same method as described above. When the sample was a solution of insulin, 2 ml of the solution was adjusted to pH 5.3–5.5, using 0.01 N hydrochloric acid or 0.1 N sodium hydroxide, and allowed to stand at 4°C overnight to permit precipitation of insulin, followed by amino acid analysis, as described above. In the case of protamine-containing preparations, the sediment obtained by centrifugation of the sample was washed several times with 0.01 M zinc chloride and then analyzed for amino acid composition.

RESULTS

Method for Purity Test: Electrophoretic patterns of the standard preparation applied at various concentrations are shown in Fig. 1. As can be seen, there was a progressive increase in density of the bands formed with increasing concentration of the standard solution, indicating this to be a practical method of assay. This fact was confirmed with use of densitometer, too.

An attempt was made to quantitate contaminants in commercial insulin preparations by electrophoretic analysis. The electrophoretic patterns are shown in Figs. 2 and 3, and the results of assays in Table 1. In addition to contaminants, proinsulin, arginineinsulin and desamidoinsulin were identified by the methods prescribed in our previous paper (5).
As evident from these data, proinsulin-like substances, which are considered to be directly related to the production of antibodies to insulin (3, 5, 6), were demonstrated in an amount of approx. 0.31%, in 11 preparations. These were absent in six preparations (A, J, K, L, N, and Q). Arginine-insulin was demonstrable in practically all the preparations tested, the content being generally similar, though relatively high in two preparations (D and M). This insulin was not detectable in a preparation (L) which is a monocomponent insulin. The deamido-insulin content varied substantially among the preparations, possible due to different degrees of formation during storage. The crystalline suspension preparations (A to M), which are less susceptible to deamidation, showed low deamido-insulin content,
whereas solutions (N, O, and P) proved to contain deamido-insulin at levels as high as 50%. The preparation (Q), which is a solution with nearly neutral pH, showed a relatively low deamino-insulin content. These findings support our previous findings that acid solutions were most susceptible to deamidation, followed, in that order, by neutral solutions and suspensions (5).

**Method for Identification of Animal Source**

The amino acid composition of bovine insulin determined by Sanger and Thompson (7) and that of porcine insulin was reported by Brown et al. (8) are compared in Table 2. These data show that the composition differs in molar ratio among the four structural amino acids, threonine (Thr), alanine (Ala), valine (Val), and isoleucine (Ile). This suggests that it might be feasible to distinguish between bovine and porcine by comparing the molar ratios of these four amino acids.

In order to determine the optimal time for hydrolysis of insulin, solutions of porcine monocomponent insulin in 6 N hydrochloric acid were hydrolysed for 24, 36, or 48 hr and assayed for molar ratio of Thr, Ala, Val, and Ile to phenylalanine (Phe) with an amino acid autoanalyzer. The results are presented in Fig. 4. As there was little or no difference in

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**Table 1.** Quantitative results on impurities in various commercial insulin preparations

|     | Arginyll insulin (%) | Proinsulin-like substance (%) | Desamidoinsulin (%) |
|-----|----------------------|-------------------------------|----------------------|
| A   | 0.31–0.63            | N.D.                          | 3.75                 |
| B   | 0.63                 | 0.31                          | 3.75                 |
| C   | 0.63–1.25            | N.D.                          | 2.50–3.75            |
| D   | 2.50                 | 0.31                          | 3.75–5.00            |
| E   | 0.31                 | 0.31                          | 2.50                 |
| F   | 0.31                 | 0.31                          | 2.50–3.75            |
| G   | 0.31                 | 0.31                          | 2.50–3.75            |
| H   | 0.31                 | 0.31                          | 2.50–3.75            |
| I   | 0.31                 | 0.31                          | 0.63–1.25            |
| J   | 0.31                 | N.D.                          | 2.50–3.75            |
| K   | 0.63–1.25            | N.D.                          | 1.25–2.50            |
| L   | N.D.                 | N.D.                          | 0.63                 |
| M   | 2.50–3.75            | 0.31                          | 3.75–6.25            |
| N   | 0.31–0.63            | N.D.                          | 60                   |
| O   | 0.31–0.63            | 0.31                          | 50                   |
| P   | 0.31–0.63            | 0.31                          | 60                   |
| Q   | 0.63–1.25            | N.D.                          | 3.13                 |

Each value indicates the mean of three determinations. The preparations (A–M) were the suspended and (N–Q) were the dissolved.
TABLE 2. Amino acid composition of bovine and porcine insulins. There are differences in the four underlined amino acids.

|       | Beef | Pork |
|-------|------|------|
| Lys   | 1    | 1    |
| His   | 2    | 2    |
| Arg   | 1    | 1    |
| Asp   | 3    | 3    |
| Thr   | 1    | 2    |
| Ser   | 3    | 3    |
| Glu   | 7    | 7    |
| Pro   | 1    | 1    |
| Gly   | 4    | 4    |
| Ala   | 3    | 2    |
| Cys   | 3    | 3    |
| Val   | 5    | 4    |
| Ile   | 1    | 2    |
| Leu   | 6    | 6    |
| Tyr   | 4    | 4    |
| Phe   | 3    | 3    |

Fig. 4. Effect of the hydrolysis term of insulin on four amino acids molar ratios. There was no difference in each amino acid molar ratio.

Fig. 5. Relationship on four amino acids, alanine (○—○), threonine (●—●), valine (△—△), and isoleucine (×—×), between molar ratios and mixture ratios when bovine and porcine insulins were combined in various proportions. All four amino acids showed linear correlation.
the molar ratios among the groups, it was decided to heat the solution for 24 hr for hydrolysis in the following experiments.

In view of the fact that many insulin preparations consist of a mixture of bovine and porcine insulin, an attempt was made to investigate if the composition of Thr, Ala, Val, and Ile might change when bovine and porcine insulins were combined in various proportions, thus distinguishing such mixtures from non-mixed preparations. Molar ratios and mixing ratios for individual preparations are plotted in Fig. 5. As can see, all four amino acids, Thr, Ala, Val, and Ile, showed linear correlations, indicating the feasibility of determining the ratio of bovine and porcine insulin in mixtures, by extrapolation.

The results of the tests for species of animal sources conducted with various commercial preparations of insulin currently available in Japan by the assay method described above are depicted in Table 3. The abbreviations and symbols are identical to those used in the study on the purity test. This test revealed that preparations consisting solely of porcine insulin, which is considered to have low antigenicity, were surprisingly few, viz., three preparations (L, M, and Q). The bovine:porcine insulin ratio of two preparations (E and I), which are said to have a ratio of 7:3, were found to 8:2.

### DISCUSSION

The results of our determination study of purity indicate that this method allows for fractional assays of impurities in various pharmaceutical insulin preparations, especially those contaminants which are antigenic (4) or cause loss of biological potency (personal
communication), and therefore is of practical significance.

The other hand, the animal source of pharmaceutical insulin preparations and the ratio of bovine and porcine insulin in a mixed preparations could be determined by the method herein described. There have been numerous clinical reports that the therapeutic effect of insulin diminishes as a result of an increase on bovine insulin-binding antibody in diabetic patients on bovine insulin for a prolonged period and that the effect is restored to normal by subsequent use of porcine insulin (9, 10). It is of great clinical significance to identify the species of animal source of pharmaceutical insulin preparations in these circumstances, and our devised assay method should be applicable for such identification.

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