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1. Masaharu Yamagishi, Toru Masuda, Makoto Yokoo, Mitsuko Asai, and Satoru Kuwada : Application of Chromatography. XXII.* Chemical Assay of Insulin Preparations.

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The purity of insulin has so far been determined by estimating its activity to depress blood sugar level in rabbits, and a product 1 mg. of which shows 22 International Units is regarded as pure. This method, however, requires skill and takes a long time. The authors undertook the present study for the purpose of finding a process which can be conducted more rapidly with exact result.

Column and paper partition chromatographies have often been applied for studying amino acids constituting insulin, or their peptide combinations, but there were only a few reports[1~3] in which a chromatographic method was employed for separation or quantitative determination of insulin itself.

Porter[1] subjected various insulin preparations to partition chromatography, using a column made by imbuing Hyflo Super-Cel with silane, and various combinations of ethyl or butyl cellosolve, potassium phosphate, and phosphoric acid as the developing solvent, and determined the quantity of insulin from the extinction (E) at 275~280 mμ of the eluted insulin fraction. With a preparation of 21.4~26.2 I.U./mg., a solution of 1 mg./cc. is said to show $E_{1\,cm.} = 1.0$.

Robinson and Fehr[2] developed protamine zinc-insulin on a paper strip, using acetic acid-butanol-water (1 : 3 : 4) as the solvent and, revealing with bromocresol green, they found protamine on the starting line and insulin on the site corresponding to Rf 0.43~0.45. The insulin band was cut out and extracted with a solvent prepared by diluting a mixture of 50 cc. of 0.1 *M* boric acid, 50 cc. of 0.2 *M* KCl, and 26.7 cc. of 0.2 *N* NaOH with water to 200 cc. The extinction at 620 mμ of the extract was measured, and the quantity of the insulin was estimated by comparing the extinction with that of a standard insulin solution.

Of late, Asaoka and Higashi[3] applied the method of Robinson, *et al.* for crude insulin and detected, besides the bands of protein and insulin, a band near that of insulin, which gave the same color reaction as insulin but had not the activity of insulin. Each of the bands was cut out and extracted, and the potency of the extract was estimated.

The authors tried the following process in order to determine insulin more rapidly and more exactly.

Insulin was separated by paper electrophoresis, the insulin band was cut out and

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* Part XXI : J. Pharm. Soc. Japan, **74**, 1347(1954).

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1) P. R. Porter : Biochem. J., **53**, 320(1953).

2) F. A. Robinson, K. L. A. Fehr : *Ibid.*, **51**, 298(1952).

3) T. Asaoka, K. Higashi : J. Pharm. Soc. Japan, **74**, 788(1954)(in Japanese).

eluted with dilute hydrochloric acid, the nitrogen in the eluate was determined by azotometric method, and the quantity of the insulin was calculated from the value of nitrogen. In this case, however, it is important to know whether the separated insulin is contaminated by other peptide compounds. Therefore, a preparation, whose insulin value had been estimated by biological assay, was subjected to the same procedure as above, and the resultant nitrogen value was compared with the bioassay value.

First of all, conditions for paper electrophoresis were determined as follows. Sample: Zinc-Insulin Crystals of Armour Laboratories, U. S. A. (23 I.U./mg.); apparatus: That previously reported by Kuwada and Masuda;[4] paper: Toyo filter paper No. 131, 2×44 cm.; electric pressure: 300 v; time: 5 hours; detection: Bromophenol blue (B.P.B.) according to the Durrum method.[5]

When 250~350 γ of the sample was migrated in Theorell buffer solution of pH 9~11 under the conditions mentioned above, insulin was separated out fairly well as shown in Table I, and when the ionic strength was lowered from 0.2 to 0.07, the separation was more complete as shown in Table II. The figures in the tables indicate the distance and the marks + and − show direction toward electrodes.

Table I. Distance in Electrophoresis (cm.)

Buffer soln.		Protein	Insulin
Theorell (ionic strength 0.2)	pH 6.0	−1	0
〃 (〃)	〃 8.0	0	+1
〃 (〃)	〃 9.0	0	+3
〃 (〃)	〃 11.0	0	+3

Table II. Distance in Electrophoresis (cm.)

Buffer soln.		Protein	Insulin
Theorell (ionic strength 0.2)	pH 9	0	+3
〃 (〃 0.07)	〃 9	0	+4
〃 (〃 0.2)	〃 11	0	+3
〃 (〃 0.07)	〃 11	0	+4.5

As mentioned before, Asaoka, *et al.*[3] subjected crude insulin to paper partition chromatography (P.P.C.), using acetic acid-butanol-water (1 : 3 : 4) as the solvent and found three bands detectable with bromocresol green (B.C.G). The authors' duplication with crude insulin of 6 I.U./mg. also gave the same result, but paper electrophoresis of the same sample gave only two bands, one on the starting line and the other on another site. The authors, therefore, conducted the following experiment to find out what pherogram the insulin-like substance reported by Asaoka, *et al.* would show. The crude insulin of 6 I.U./mg. was developed on a starch column, using the same solvent as in the above P.P.C. and eluted, dividing the eluate into fractions of 10 cc. each. The first three fractions were positive to B.P.B. but the first was most positive. When the first fraction was subjected to P.P.C., two bands detectable with B.P.B. were found side by side, but no spot on the starting line. The same sample was then subjected to paper electrophoresis using Theorell buffer, when one spot on the starting line and another on the site corresponding to the insulin site were detected by B.P.B. From the result it is evident that the substance of Rf 0.40 reported by Asaoka, *et al.* remained on the starting line during the electrophoresis. Consequently, it may be safely said that the substance migrated in the electrophoresis must be pure insulin.

A solution was prepared by dissolving 45.1 mg. of the preparation of the Armour Laboratories in 1.2 cc. of 0.05 *N* HCl, and 0.05 cc. of this solution (equivalent to 1.88 mg. of the sample) was applied on paper and developed under the conditions mentioned above.

4) S. Kuwada, T. Masuda : Vitamins, **6**, 97(1953)(in Japanese).
5) E. L. Durrum : J. Am. Chem. Soc., **72**, 2943(1950).

The insulin band was cut out and eluted with 10 cc. of water, the eluate was decomposed by the micro-Kjeldahl method, and the nitrogen of the decomposition product was determined by azotometry. On the other hand, it was ascertained that the filter paper contained no nitrogen, and that the elution was 100% from agreement between the nitrogen-value obtained by the direct determination and that obtained by applying the sample on paper, then eluting the sample without being subjected to electrophoresis, and finally determining nitrogen in the eluate (Table III).

Table III. Azotometry of Eluate of Insulin-Band

Treatment of sample	N value from sample soln. of 2 cc. (γ)	N value in original sample (%)
After electrophoresis the insulin band was eluted	226.5	12.04
〃	228.0	12.10
After placing the insulin band was eluted without elctrophoresis	268.5	14.28
〃	263.5	14.01

The nitrogen contents of original samples were determined by micro-Dumas method.

Sample 3.145 mg., N_2 0.4224 cc. (20°, 765 mm.) N%=14.36
〃 3.200 〃 , 〃 0.3900 cc. (21°, 764 mm.) 〃 =14.23

After the treating of same samples with micro-Kjeldahl method, the nitrogen contents were determined by azotometric method.

Sample 382 γ, N_2 volume (mm^3) 42.9, N%=14.05
〃 〃 〃 43.6, 〃 =14.27

From the above results it was found that the insulin was separated by electrophoresis from a small amount of protein-like impurities having different electric charge.

Various insulin preparations which had been tested by biological method were subjected to electrophoresis under the same conditions, and the nitrogen-values of the insulin bands were determined as above. The results are shown in Table IV. In this case,

Table IV. Comparison of Biological Assay (I. U.) and Chemical Assay (N content)

Sample No.	Quantities of sample, placed on the paper strip (γ)	Quantities of N, found by azotometry (γ)	Quantities of N, calcd., for the sample on the paper (γ)	Quantities of N, calcd., for 1 mg. insulin prep. (γ)	I. U./mg. estimated biologically**	Quantities of N, calcd. for 1 I. U.
1	505	25.5	63.7	126.2	25.9	4.9
〃	520	23.2	58.0	111.5	〃	4.3
2*	1880	45.5	227.5	120.8	23.0	5.3
3	502	21.2	53.0	105.5	22.0	4.8
4	500	18.0	45.0	90.0	18.0	5.0
5	502	14.2	35.5	70.0	15.4	4.55
〃	〃	16.2	40.5	80.5	〃	5.2
〃	545	18.2	45.5	83.4	〃	5.4
6	330	8.8	22.0	66.5	15.2	4.4
7	518	9.4	23.5	45.3	6.3	7.2
8	3600	34.3	85.5	23.8	4.8	5.0

Sample : Nos. 1 and 2—Insulin Crystals of Armour Laboratories, U. S. A.
Nos. 3 and 4—Insulin Crystals of Shimizu Pharm. Co.
Nos. 5, 6, 7, and 8—Amorphous (crude) insulin of Shimizu Pharm. Co.

* 2 cc. of the eluate was employed.

** I. U. in this table was determined by the respective companies which produced the preparations.

10 mg. each of the preparations was dissolved in 1 cc. of Theorell buffer solution (ionic strength, 0.07), and 0.05 cc. of the solution was applied on paper, 2 × 42 cm. (a), and about 0.025 cc. on another paper, 2 × 42 cm. (b), and both were developed under the same conditions, using the same buffer solution. The insulin band on the paper (a) was located from the insulin position of the paper (b) detected by B.P.B. The insulin band on the paper (a) was cut out, eluted with 10 cc. of warm water for 30 minutes, and 4 cc. of the eluate was treated as above to determine the nitrogen-value in it.

According to the results of biological assay, 1 I.U. corresponds to 4.3~5.5 γ of nitrogen. With samples of low potency, however, the present method gave larger nitrogen values against the international unit of the samples. It may be that biological assay gives lower values than actual ones with increase of protein-like impurities.

More data are necessary for ascertaining the accuracy of the present method, but the authors' belief is that insulin preparations with at least 15 I.U. could be determined by the present chemical method.

The authors express their thanks to the Shimizu Pharmaceutical Company for their donation of various samples of insulin preparations.

Summary

When subjected to paper electrophoresis under the present conditions (buffer, Theorell buffer solution of pH 9~11; ionic strength, 0.07; electric pressure, 300 v; time, 5 hours) insulin migrated towards the anode by ca. 4 cm., forming a clear band. The insulin band was located by duplicate method, cut out, and eluted with water. The eluate was decomposed by the micro-Kjeldahl method and nitrogen in the product determined by azotometry. From the comparison of the results with those of biological assay it was concluded that the present chemical method is applicable for determining the potency of insulin preparations.

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2. Tanezo Taguchi and Masaharu Kojima : Studies in Stereochemistry. IV.[1] Alkanolamines. (4). Regular *dl*-2,5-Diphenyl-4-methyloxazoline : The Formation and Action of Methyl Tosylate.

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Of the two forms of *dl*-2,5-diphenyl-4-methyloxazoline, the ψ-form (VII) has been obtained[2] but not the regular form (VI). It has been found[2] that the action of concentrated sulfuric acid on *dl*-ψ-N-benzoylnorephedrine does not give the regular *dl*-oxazoline but the *dl*-ψ-oxazoline with retension. Therefore, the formation of the regular *dl*-oxazoline (VI) was studied and it was found to be accomplished by two methods. In addition, the regular *dl*-oxazoline (VI) was treated with methyl tosylate in order to see the feasibility of this method for N-monoalkylation of β-aminoalcohols, reported in the previous papers.[1,3,4]

The formation of the regular *dl*-oxazoline (VI) was successfully attained by two methods, one being based on the reaction between *dl*-norephedrine and benzimidoethyl ether hydrochloride,[5] and the other, the boiling of *dl*-ψ-1-phenyl-1-chloro-2-benzoylaminopropane (IV) in absolute ethanol containing anhydrous sodium carbonate. *dl*-ψ-1-Phenyl-1-chloro-2-benzoylaminopropane (1V) is prepared by the benzoylation of *dl*-ψ-1-phenyl-1-chloro-2-aminopropane (III) which is obtained without the accompaniment of the other racemic isomer on the treatment of either *dl*-norephedrine (I) or *dl*-ψ-nor-

* Katakasu, Fukuoka (田口胤三, 小島正治).

1) Part III : J. Pharm. Soc. Japan, **74**, 1293(1954).
2) N. Nagai, S. Kanao : Ann., **470**, 157(1929).
3) T. Taguchi, M. Kojima : This Bulletin, **1**, 325(1953).
4) T. Taguchi, M. Kojima : J. Pharm. Soc. Japan, **74**, 1133(1954).
5) At the Annual Meeting of the Pharmaceutical Society of Japan (1954) it was reported that the reaction was unsuccessful but later prolongation of reaction time allowed successful completion.