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Stability of the 4th International Standard for Insulin

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Summary. The stability of the (W.H.O.) 4th International Standard for Insulin, has been assessed by accelerated thermal degradation studies. This is a crystalline preparation of insulin, freed from proteolytic enzymes, sealed in ampoules containing air and with a moisture content of 5–6%. Of the original biological activity 95.8 (92.8–98.9; P = 0.95)% was retained after storage for 12 years in the dark at 20° C and 65.7 (63.4–68.1; P=0.95)% after 14 years at 37° C. Degradation rate constants were calculated from these data for the Standard at 20° C and 37° C, assuming first order kinetics. The degradation constant at 37°C did not differ significantly from those found in earlier degradation studies at 37°C over shorter periods, thereby supporting the assumption that the degradation of crystalline insulin, at least at 37° C, is a first order reaction. Extrapolation of these data suggests that the Standard stored at –20°C for 20 years would have retained at least 99.93% (P = 0.95) of its original activity and so for practical purposes can be considered to be stable.

Key words: Insulin standard, insulin stability, insulin accelerated thermal degradation, insulin biological activity.

Earlier studies [1–7], have investigated the stability of insulin in solution or suspension. This report describes the stability of crystalline insulin.

The (W.H.O.) 4th International Standard for Insulin (4th I. S. Insulin) was sealed in 1955 in glass ampoules containing air (of approximately constant humidity during the filling) and with an average moisture content of 5.65% [8]. This differs from the procedure customarily followed for the preparation of international standards, which are generally dried to a final moisture content of less than 1% and sealed under dry nitrogen. Preliminary accelerated degradation studies were performed in 1961 with samples kept at 37° C for 25 months, and in 1963 with samples kept at 37° C for 49 months [9].

The present study has been carried out to compare the activity of degradation samples kept at 37°C and 20°C for 14 and 12 years, respectively, with the Standard, which has been stored at –20°C since 1966 and previously at –10°C. The data from all these degradation studies have been extrapolated to assess the stability of the Standard under the conditions at –20°C in the dark, in which it is normally kept.

Materials and Methods

Four samples were included in the study and coded: (A) 4th I. S. Insulin stored at 37° C for 14 years; (B and C) two samples of 4th I. S. Insulin, stored at −20° C since 1966, and previously at −10° C; and (D) 4th I. S. Insulin stored at 20°C for 12 years. Some difficulty was experienced in solubilizing (A), but solution was complete at the dilutions used for assay.

Three of the four invited laboratories participated in the study, and these are listed in the Annexe, but the order in which they appear is not necessarily that of the code numbers allocated to them. Two assay methods were used, the mouse convulsion method [10] (laboratories 1 and 2) and the rabbit blood-sugar method [11] (laboratories 2 and 3). Each participant was asked to compare sample A with B and sample C with D to a precision of not less than ± 10% for each assay method. It was suggested to the participants that the assumed relative potencies be adjusted for each assay, to avoid bias of the estimated potency by the assumed potency.

Results

Two laboratories did not provide sufficient information for detailed statistical analysis of individual assays to be carried out. It was therefore decided to use relative potencies, calculated by each laboratory from their own data, as the starting point for the analysis described below. Where necessary, relative potencies were adjusted so that activity of a degradation sample (A or D) was expressed as a percentage of the Standard, (B or C). Laboratory 3 did not compare preparations in the order requested, but assayed each of the four samples against their own supply of the 4th I. S. Insulin. Potencies thus obtained for B and C did

Table 1. *Combined potencies for sample A (37°C for 14 years) as a percentage of sample B (Standard)*

Laboratory Number	Method					
	Mouse Convulsion			Rabbit Blood Sugar		
	Potency	Fiducial Limits ($P = 0.95$)	Weight	Potency	Fiducial Limits ($P = 0.95$)	Weight
1.	62.9	(57.5–68.8)	2,528		–	
2.	61.8	(58.4–65.4)	6,310	66.8	(61.9–72.2)	3,533
3.		–		73.8	(68.4–79.5)	3,723
All labs.	62.1	(59.2–65.2)	8,838	70.3	(66.6–74.2)	7,256

Potency combined for all laboratories and both methods is 65.7, with fiducial limits ($P = 0.95$) of 63.4 and 68.1, and an assay weight of 16,094

Table 2. *Combined potencies for sample D (20° C for 12 years) as a percentage of sample C (Standard)*

Laboratory Number	Method					
	Mouse Convulsion			Rabbit Blood Sugar		
	Potency	Fiducial Limits ($P = 0.95$)	Weight	Potency	Fiducial Limits ($P = 0.95$)	Weight
1.	98.0	(90.2–106.6)	2,919		–	
2.	98.3	(93.0–103.8)	6,677	91.2	(85.7–97.1)	5,426
3.		–		96.2	(90.5–102.2)	5,687
All labs	98.2	(93.8–102.8)	9,596	93.7	(89.7–97.9)	11,113

Potency combined for all laboratories and both methods is 95.8, with fiducial limits ($P = 0.95$) of 92.8 and 98.9, and an assay weight of 20,709

Table 3. *Homogeneity of log potencies*

Comparison	Method	Number of Assays	χ^2	P	Mean Potency
Sample A (37°C for 14 years) v Sample B (Standard)	Mouse Convulsion	11	8.4	0.5–0.7	62.1%
	Rabbit Blood Sugar	8	4.8	0.5–0.7	70.3%
	All	19	24.7	0.1–0.2	65.7%
Sample D (20°C for 12 years) v Sample C (Standard)	Mouse Convulsion	13	14.8	0.2–0.3	98.2%
	Rabbit Blood Sugar	10	8.9	0.3–0.5	93.7%
	All	23	25.9	0.2–0.3	95.8%

not differ significantly from 100%; therefore the potencies estimated by this laboratory for A and D were included with those obtained by the other two laboratories in direct comparison with the Standard. Laboratory 2 provided three independent potency estimates for B and C by use of the mouse convulsion method. Each of these potencies had been derived from 10–20 individual assays as described in the British Pharmacopoeia [10].

Each log potency was weighted by the reciprocal of its variance and by use of χ^2 it was shown that estimates obtained within each laboratory and each assay method formed a homogeneous set. Log potencies were therefore combined within these sub-sets and weighted means, together with fiducial limits, are given in Tables 1 and 2. The assay method in rabbits appeared to give higher estimates of potency for the 37°C sample than the method using mice and the reverse was true for the 20°C sample. However, log potencies obtained by both methods were shown to be homogeneous for each sample (Table 3) and further combination of results was considered justified, giving final estimates of 65.7% activity for the 37°C sample and 95.8% activity for the 20°C sample.

The logarithm of the degradation constant (log K) was calculated [12] for 20°C and 37°C from the combined potency estimates for each method and laboratory (Table 4, Study 3). The linear regression of log K on 1/T (the reciprocal of absolute temperature) was then computed from these values, together with the 95% confidence limits of the slope. By extrapolation of this regression line and the lines obtained from the confidence limits the value of log K at −20°C was estimated to be −12.91 with limits of −14.34 to −11.49.

Another estimate of log K at −20°C was made by using results from all three degradation studies. In each of two earlier studies [9] in 1961 and 1963, respectively, coded solutions were prepared, by the same person on the same day and in an identical manner, from the 4th I. S. Insulin (then stored at −10°C) and from degradation samples kept at 37°C for 25 and 49 months, respectively. These samples were assayed by the mouse convulsion method, by three laboratories in the first study and four laboratories in the second. The data obtained in the first study [9] gave a potency estimate combined for all laboratories, and for the second study combined potency estimates for each laboratory (see Table 4). These combined potency estimates were used to calculate the log K values shown in Table 4. A regression line, and its 95% confidence limits were then calculated from the log K values of all three studies. The additional data from the two earlier studies influenced only the calculation of the degradation constant at 37°C; as a result the slope of the regression line was little different, but the 95% confidence limits were narrowed by inclusion of the extra information. By extrapolation, log K at −20°C was calculated to be −12.89, with 95% confidence limits of −13.87 and −11.91. The calculations suggest that the potency of the 4th I. S. Insulin, when stored at −20°C for 20 years, would have retained 99.98% of its original activity, with 95% confidence limits of 99.93% to approximately 100%.

Discussion and Conclusions

The present accelerated degradation studies on the 4th I. S. Insulin have shown that 95.8%, with fiducial limits ($P = 0.95$) of 92.8% and 98.9%, of the

Table 4. *Rate constant values obtained from degradation studies of 1961, 1963 and 1973*

Study Number	Laboratory Number	Method[a]	Temperature °C	Time	Remaining Activity (% of original)	Log K
1.(1961)	All (3) labs	MC	37	25 months	94.00	−9.03
2.(1963)	1	MC	37	49 months	84.14	−8.87
	2	MC	37	"	88.46	−9.02
	3	MC	37	"	94.35	−9.35
	4	MC	37	"	86.76	−8.96
3.(1973)	1	MC	37	14 years	62.89	−8.97
	2	MC	37	"	61.82	−8.96
	2	RBS	37	"	66.83	−9.03
	3	RBS	37	"	73.78	−9.16
	1	MC	20	12 years	98.04	−10.25
	2	MC	20	"	98.27	−10.30
	2	RBS	20	"	91.23	−9.59
	3	RBS	20	"	96.18	−9.96

[a] Mouse convulsion method (MC) and rabbit blood sugar method (RBS)

original biological activity is retained after 12 years storage in the dark at 20°C and 65.7%, with fiducial limits ($P = 0.95$) of 63.4% and 68.1%, after 14 years in the dark at 37°C.

These data have been used to calculate the degradation rate constants, assuming first order kinetics, for the Standard at 20°C and 37°C. The degradation rate constant at 37°C, computed from these data, does not differ significantly from those found in earlier degradation studies at 37°C over shorter periods. Since the constants computed for different time intervals show such good agreement, the assumption that the degradation of crystalline insulin is a monomolecular reaction, at least at 37°C, receives confirmation. First order kinetics for insulin degradation in solution have earlier been described [1, 7].

Extrapolation of these data gives an estimate of the degradation rate constants for the Standard stored at −20°C. This suggests that the Standard stored at −20°C for 20 years would have retained at least 99.93% of its original activity ($P = 0.95$), and for practical purposes can therefore be considered to be stable.

The great stability of this crystalline insulin may in part be due to the particular attention given during its preparation to the removal of contaminating proteolytic enzymes [8].

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ANNEXE

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