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The proinsulin content of commercial bovine insulin formulations

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The proinsulin-like immunoreactivity (PLI) content of traditional and highly purified bovine insulin preparations sold by three insulin manufacturers in the UK has been estimated by radioimmunoassay. We report the PLI content of 22 batches of traditional insulin products and 32 of highly purified insulin, marketed between March 1980 and March 1983. Estimates of the PLI content of traditional insulins ranged from 2290 to 48400 parts per million (ppm). In contrast the estimated PLI content of the highly purified insulins varied between less than 1 ppm and 1160 ppm, although more recent batches prepared by current methodology contained less than 10 ppm of PLI. We conclude that highly purified bovine insulin on sale in the UK may now be expected to contain less than 10 ppm of proinsulin-like immunoreactivity.

Many patients treated with 'conventional' non-'highly-purified' insulin develop anti-insulin antibodies soon after beginning treatment. Many factors, including the species of origin and purity of the insulin preparation contribute to this immune response (for review see Reeves 1980). Proinsulin has been considered to be one of the major immunogenic contaminants of such products and transfer of patients treated with them to highly purified porcine insulin containing less than 1 part per million (ppm) proinsulin, results in a fall in both anti-proinsulin and anti-insulin antibodies (Heding et al 1980).

Since 1980, preparations of bovine insulin described as 'highly purified' have been marketed in the United Kingdom by British manufacturers and have been widely used. Little information has been reported on the degree of proinsulin contamination of these insulins, making it difficult to interpret results of studies on the effects of transfer of patients to highly purified bovine insulins. In this study we report the proinsulin-like immunoreactivity (PLI) content of 22 batches of traditional bovine insulin preparations and 32 batches of highly purified bovine insulin sold in the United Kingdom.

MATERIALS AND METHODS

1 *Immunoassay reagents*

Bovine proinsulin was obtained from Novo Research Institute, Denmark.

Iodinated tracer was prepared as follows: bovine proinsulin (10 μg) was iodinated with 1·0 mCi of Na ^{125}I (Amersham, UK) according to the method of Hunter & Greenwood (1962). The products of the reaction were separated by reversed-phase high performance liquid chromatography (hplc) using an Altex series 342 liquid chromatograph (Anachem Ltd, Luton, UK). Chromatography was performed on columns (10 × 0·46 cm) of Nucleosil C-8 (Jones Chromatography Ltd, UK) in 0·1% trifluoroacetic acid using a linear acetonitrile gradient of 16% acetonitrile to 52% acetonitrile in 40 min. Two peaks of radioactivity were routinely observed, the major peak eluting 7 min after unmodified bovine proinsulin. This peak, which also exhibited the highest affinity for the anti-proinsulin antiserum (see below) was collected, freeze-dried in the presence of human serum albumin and stored in aliquots at −40 °C for up to two months.

Anti-bovine proinsulin antiserum was prepared as follows. Six adult male guinea-pigs were each injected with 40 μg of bovine proinsulin emulsified with Freund's complete adjuvant, at multiple intradermal sites. Subsequent injections were carried out at three-week intervals and consisted of 40 μg per animal given intraperitoneally with Freund's incomplete adjuvant.

After five injections the animals were bled out. The serum exhibiting maximum binding of labelled proinsulin was further treated to remove anti-insulin activity. Pure porcine insulin (Novo Research Institute) was reacted with Cyanogen-bromide activated sepharose-4B (Pharmacia Fine Chemicals, Sweden) using the methods recommended by Pharmacia, to give a final ratio of 10 mg insulin per ml of gel. The antisera were treated with this insulin-sepharose (1 ml antiserum ml^{-1} gel) for 24 h at 4 °C. Following this treatment, the insulin binding component was

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reduced to undetectable levels, leaving the anti-proinsulin antiserum. The final dilution of this antiserum used in the radioimmunoassay was 1/207 000.

2 *Radioimmunoassay of bovine proinsulin*

Radioimmunoassay of bovine proinsulin was based on the method of Heding et al (1974). Briefly, immunoassay tubes contained 100 µl of NaFAM buffer (NaCl, 6·0, NaH_2PO_4 $2H_2O$, 1·18, $Na_2HPO_4 \cdot 12H_2O$, 12·16 thiomersal, 0·24, human serum albumin, 10·0 g litre^{-1}, pH 7·32) and 100 µl of 40 u ml^{-1} insulin solution to be tested (soluble or neutral formulations) or 100 µl proinsulin solution (0·1–2·4 ng/100 µl). In those tubes containing proinsulin standards, or when the proinsulin content of the insulin preparation was high, necessitating dilution, 40 u ml^{-1} of Nordisk porcine insulin (Velosulin) was used as a diluent, thus ensuring that each tube contained 100 µl of 40 u ml^{-1} insulin. Pure porcine insulin (40 u ml^{-1}, Nordisk, Denmark) gave zero displacement of tracer in this assay. In those experiments where 80 u ml^{-1} insulin was assayed, 80 u ml^{-1} Nordisk insulin was used as a diluent.

To each tube, containing 200 µl of insulin in NaFAM buffer as described above, was added 40 µl of antiserum diluted in NaFAM to give a final concentration of 1/207 000. Following incubation at 4 °C for 4 h, 50 µl of [^{125}I]proinsulin (10 000 counts min^{-1} in NaFAM buffer) was added. Tubes were incubated at 4 °C for a further 16 h. Bound tracer was precipitated by the addition of 1·6 ml of 96% ethanol at 4 °C. After centrifugation, the precipitate was washed once with 2 ml of a solution containing 960 ml 96% ethanol, 162 ml water and 18 ml FAM buffer (0·04 M phosphate pH 7·4, 0·1% human albumin). After it had been further centrifuged, radioactivity in the pellet was measured using an LKB Multigamma.

3 *Insulin batches*

With the exception of Boots batch 91745 which was made available before licensing, all batches were obtained as part of the 'stop order' procedure associated with product licensing.

4 *Treatment of data*

All samples were analysed in at least two independent assays. Initially 5-fold dilution increments were used in the estimation of the level of PLI. Subsequently, full displacement curves were routinely determined using 1·4 fold dilution increments. Data were analysed using the WRANL program of Gaines-Das & Tydeman (1982).

5 *Polyacrylamide gel electrophoresis*

Polyacrylamide gel electrophoresis in 8·0 M urea was performed according to the method given in the British Pharmacopoeia (1980).

RESULTS

1 *Bovine proinsulin radioimmunoassay*

The preparation of bovine proinsulin used as a standard in these studies displaced iodinated proinsulin tracer at between 0·3 and 2·4 ng proinsulin/tube (Fig. 1). The sensitivity of this assay system is comparable to the previously published proinsulin radioimmunoassay of Heding et al (1974). Assuming the potency of pure insulin to be 27 u mg^{-1}, the limit of detection of proinsulin in 40 u ml^{-1} insulin was about 2 ppm and in 80 u ml^{-1} insulin about 1 ppm.

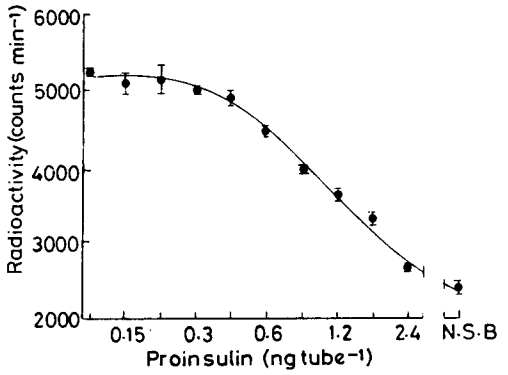

FIG. 1. Bovine proinsulin standard curve. Radioimmunoassay of bovine proinsulin was performed as described in Materials and Methods. Error bars represent the standard error of the mean of triplicate determinations. NSB = non-specific binding.

2 *Proinsulin content of formulated bovine proinsulin*

Proinsulin-like immunoreactivity (PLI) in 22 batches of traditional preparations and 32 batches of highly purified bovine insulin from three manufacturers, Boots, Wellcome and Weddel Pharmaceuticals, is shown in Tables 1 and 2. Traditional bovine insulin formulations (Table 1) from Boots and Wellcome generally contained between 15 000 and 50 000 ppm of PLI, whereas the non-highly purified bovine insulin of Weddel contained between 2290 and 9700 ppm. All traditional formulations showed a band corresponding in mobility to bovine proinsulin on gel-electrophoresis. In contrast, highly purified bovine insulin formulations (Table 2) showed very

Table 1. Proinsulin-like immunoreactivity in traditional, non-highly purified bovine insulin. PLI contents of commercial soluble or neutral bovine insulins were determined as described in Materials and Methods. Fiducial limits are at the 95% interval.

Manufacturer and batch no	Marketing date	Strength u ml^{-1}	PLI (ppm)	Fiducial limits $P = 95$
Wellcome				
6342	July 1981	40	22000	20800–23200
6344	Jan. 1982	40	28900	27000–31100
6348	April 1982	40	17300	13800–23700
6356	July 1982	40	36000	31500–40900
6350	April 1982	80	48400	38600–62000
6354	Feb. 1982	80	16700	13500–22400
6358	July 1982	80	17900	14700–21800
Weddel Pharmaceuticals				
1558A	March 1981	40	7320	6240–8620
1560A	June 1981	40	3600	3240–4030
1566A	Aug. 1981	40	3050	2860–3240
1580A	June 1982	40	3460	2860–4130
1552A	Nov. 1980	80	2290	1920–2750
1568A	Sept. 1981	80	9700	7378–14600
1582A	June 1982	80	3000	2190–3890
Boots				
92486	Dec. 1981	40	15300	14500–16000
92487	Feb. 1982	40	32300	29100–36200
92658	June 1982	40	30700	21800–40900
92661	April 1982	40	6595	4320–9490
92446	Dec. 1981	40	28990	27100–30800
92488	June 1982	40	25600	24300–27000
92628	April 1982	40	31900	26200–38600
92659	April 1982	40	35200	27600–45100

much lower levels of PLI. Wellcome highly purified bovine insulin contained between 3 and 73 ppm of PLI, although none of the 100 u ml^{-1} batches contained more than 21 ppm, and the four most recent batches contained between 3 and 7 ppm. The earlier batches of highly purified bovine insulin from Weddel were somewhat variable, one containing up to 1160 ppm. Batches marketed after December 1982, however, contained much lower levels (<10 ppm) and the three 100 u ml^{-1} formulations examined in this study contained no detectable PLI. Only two batches of highly purified Boots neutral insulin have been marketed at the time of this study, both of which contained <10 ppm.

DISCUSSION

Bovine insulin has been marketed in the UK for many years, and 'highly purified' bovine insulin has been available since 1980. Although the production of anti-insulin and anti-proinsulin antibodies has often been observed, particularly in patients treated with conventional bovine insulin (Reeves 1980), many patients have been successfully stabilized on both types of product. Monocomponent porcine insulin has been reported to contain <1 ppm of PLI (Heding et al 1980) (without independent confirmation), and the relatively low frequency of antibody production in patients on monocomponent pork insulin (Heding et al 1980), has been ascribed to the low levels of PLI.

The data presented in this study indicate that traditional bovine insulin products may contain up to 5% w/w PLI (Table 1). The non-highly purified product from Weddel tended to contain approximately 10-fold less PLI than the comparable product from Boots and Wellcome, and this product may represent an intermediate between traditional and highly purified insulins.

Table 2. Proinsulin-like immunoreactivity in highly-purified bovine insulin. PLI contents of commercial soluble or neutral highly purified bovine insulins were determined as described in Materials and Methods. Fiducial limits are at the 95% interval.

Manufacturer and batch no.	Marketing date	Strength (u ml^{-1})	PLI (ppm)	Fiducial limits $P = 95$
Wellcome				
A1022	Sept. 1980	40	32	22–44
A1030	May 1981	40	29	22–39
A1064	April 1982	40	41	36–46
A1084	June 1982	40	34	30–39
A1012	May 1980	80	28	20–42
A1018	Sept. 1980	80	49	41–71
A1032	May 1981	80	38	16–56
A1078	Aug. 1982	80	73	61–88
A1096	Oct. 1982	80	15	14–17
1134	Nov. 1982	100	19	18–21
A1102	Dec. 1982	100	16	14–19
1130	Dec. 1982	100	21	17–27
1136	Dec. 1982	100	3	2·5–3·5
1122	Dec. 1982	100	6	5–7
1124	Dec. 1982	100	7	6·5–8·5
A1150	Jan. 1983	100	5	4·6
Weddel Pharmaceuticals				
1532A	March 1980	40	229	182–300
1565A	Sept. 1981	40	857	784–935
1573A	April 1982	40	16	13–20
1583A	July 1982	40	158	137–187
175A	Jan. 1983	40	<2	<2
160A	Dec. 1982	80	7	6–8
1534A	April 1980	80	611	554–668
1561A	July 1981	80	1157	1070–1250
1569B	Nov. 1981	80	635	519–792
1579A	June 1982	80	5	4–6
163	Dec., 1982	100	<1	<1
177A	Feb. 1983	100	<1	<1
181A	March 1983	100	<1	<1
Boots				
91745	not marketed	80	18	16–22
92729	Oct. 1982	100	2	1·5–2·5
93003	Feb. 1983	100	7	6–9

Overall, the highly purified bovine insulin preparations examined in this study contained between <1 and 1160 ppm of PLI. Table 2, however, reveals that the extent of PLI contamination has decreased since the introduction of the product in 1980. Batches 1136, 1122, 1124 and A1150 from Wellcome, 175A, 160A, 163, 177A and 181A from Weddel, and 92729 and 93003 from Boots may be considered representative of current good technology for the production of bovine insulin. In each of these batches the level of PLI was <10 ppm and in most cases approached or

exceeded the lower limit of sensitivity of the assay system used. Although this study has only included soluble and neutral formulations, crystalline and isophane formulations are unlikely to contain different concentrations of PLI. Current production batches of highly purified bovine insulin, for sale in the UK may thus be expected to contain <10 ppm of PLI, although it should be noted that the 2 year shelf-life on insulin means that earlier, less pure, batches may be on sale until mid 1984.

It should be noted that the estimates of PLI by immunoassay expressed as ppm are, to a certain extent, arbitrary since there is no international standard yet for bovine proinsulin. Nonetheless, the data provide a firm indication of the relative content of the various preparations, and where independent estimates on the same batches have been available (Wellcome, Boots, personal communications) these have generally been in close agreement with our findings. It should also be noted that proinsulin is not necessarily the only antigenic contaminant in bovine insulin and in this study we have not examined such factors as high-molecular weight non-proinsulin contaminants, which are strictly controlled in some highly-purified formulations.

Although this report makes no contribution to the role of proinsulin in the immunogenicity of insulin, it is interesting to consider these findings in the light of a recent report by Peacock et al (1983) in which highly purified bovine insulin (which contained 20–40 ppm proinsulin, Boots), although less effective than pure pork or human insulin in reducing the level of anti-insulin antibodies in previously sensitized patients, elicited a significant fall in the level of anti-C-peptide antibodies, suggesting that levels of PLI lower than 20–40 ppm are sufficiently low to abolish that part of the immune response attributable to proinsulin.

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