The International Reference Preparation of Calcitonin, Human, for Bioassay:
Assessment of material and definition of the International Unit

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Abstract. An international reference material is required for bioassays of preparations of synthetic human calcitonin for administration to man and for use in immunoassays.

A preparation of synthetic human calcitonin in ampoules coded 70/234 (previously widely used as the MRC Research Standard) has been examined in an international collaborative study involving 7 laboratories in 6 countries. The results of 34 in vivo bioassays with two other preparations of synthetic human calcitonin showed that this preparation was suitable to serve as a standard.

With the agreement of the participants in the collaborative study, the batch of ampoules, code 70/234, was established in 1978 by the World Health Organization as the International Reference Preparation of Calcitonin, Human, for Bioassay. The International Unit of human calcitonin was defined as the activity contained in one ampoule of this preparation, thus maintaining continuity of the unit of the research standard.

The effectiveness of porcine and salmon calcitonin in the treatment of Paget's disease of bone has been widely recognized and certain advantages have been suggested in the use of human calcitonin for this purpose (British Medical Journal 1975). Moreover immunoassays of calcitonin in human plasma are now in use, e.g. for the diagnosis of medullary carcinoma of the thyroid (e.g. 26th Report of the ECBS 1975). Thus an international reference preparation has become necessary for bioassays of preparations of synthetic human calcitonin for clinical administration and for immunoassays.

This paper describes the International Reference Preparation of Calcitonin, Human, for Bioassay (previously the MRC Research Standard for human calcitonin in ampoules labelled 70/234 and referred to hereafter in this paper as IRP), and reports the results of an international collaborative study carried out to assess this material for its suitability to serve as a standard for bioassays.

Materials and Methods

The International Reference Preparation of Calcitonin, Human, for Bioassay

In January 1970, Ciba Ltd., Basle, Switzerland, through the good offices of Dr. W. Rittel, generously donated 30 mg of synthetic human calcitonin of the amino acid sequence of the peptide derived from thyroid medullary tumours for use as a reference preparation. The material, batch number 2076X, made by classical fragment assembly, was stated to contain 15% residue of acetic acid and water, and 85% peptide.

In March 1970, 26 mg of the powder was dissolved in 0.05 M acetic acid in double glass distilled water containing 1.0% mannitol and sterilized by membrane filtration. The solution was distributed into approximately 2500 neutral glass ampoules (code numbered 70/234) and freeze-dried. The mean weight of solution in each of 50 weighed ampoules was 1.015 g (variance ± 0.24%). After secondary desiccation, the ampoules were filled with pure dry nitrogen and sealed by glass fusion (procedures described in the 29th Report of the ECBS, Annex 4
Table 1. Details of laboratory methods.

<table>
<thead>
<tr>
<th>Lab. No.</th>
<th>Route of injection</th>
<th>Strain, sex and weight of rat</th>
<th>Diluent and carrier protein</th>
<th>Interval between injection and sample (min)</th>
<th>Method of blood sampling</th>
<th>Method of calcium analysis in plasma or serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Subcutaneous</td>
<td>Local Male 150–175 g</td>
<td>Saline pH 3.2 1% gelatine</td>
<td>60</td>
<td>Abdominal aorta</td>
<td>Serum Autoanalyser</td>
</tr>
<tr>
<td>2</td>
<td>Intravenous</td>
<td>Local Female 100 g</td>
<td>Sodium acetate pH 4.6 0.1% bovine serum albumin</td>
<td>50</td>
<td>Retro-orbital venous plexus</td>
<td>Plasma Atomic absorption spectrophotometry</td>
</tr>
<tr>
<td>3</td>
<td>Intravenous</td>
<td>Sprague Dawley Male 45–65 g</td>
<td>Sodium acetate pH 4.0 0.2% bovine serum albumin</td>
<td>60</td>
<td>Cardiac puncture</td>
<td>Serum Atomic absorption spectrophotometry</td>
</tr>
<tr>
<td>4</td>
<td>Subcutaneous and intravenous</td>
<td>Sprague Dawley Male 45–55 g</td>
<td>Saline pH 3.5 0.1% human serum albumin</td>
<td>60</td>
<td>Retro-orbital venous plexus</td>
<td>Serum Fluorimetric</td>
</tr>
<tr>
<td>5</td>
<td>Intravenous</td>
<td>Sprague Dawley Male 90–100 g</td>
<td>Sodium acetate pH 4.0 0.1% bovine serum albumin</td>
<td>60</td>
<td>Severing carotid artery and jugular vein</td>
<td>Serum Atomic absorption spectrophotometry</td>
</tr>
<tr>
<td>6</td>
<td>Intravenous</td>
<td>Sprague Dawley Male 45–55 g</td>
<td>Sodium acetate pH 4.0 0.1% bovine serum albumin</td>
<td>60</td>
<td>Cardiac puncture</td>
<td>Plasma Atomic absorption spectrophotometry</td>
</tr>
<tr>
<td>7</td>
<td>Subcutaneous</td>
<td>Wistar Male 100 g</td>
<td>Sodium acetate pH 4.0 0.1% bovine serum albumin</td>
<td>60</td>
<td>Abdominal aorta</td>
<td>Serum Atomic absorption spectrophotometry</td>
</tr>
</tbody>
</table>
(1978)). The ampoules have since been stored at −20°C in the dark.

The nominal content of each ampoule is approximately 8.5 μg human calcitonin peptide and 10 mg mannitol.

This ampouled material was established in 1970 as the Medical Research Council Research Standard for calcitonin, human, with an assigned unitage of 1.0 unit/ampoule. It has since been used by research workers for bioassays and immunoassays.

Other preparations included in the collaborative study

Two recently synthesized batches of human calcitonin from different sources, ampouled to the same specifications as the International Reference Preparation, were also included in the study.

A preparation of human calcitonin made by classical fragment assembly (internal Batch No. Labor Los 424 m Lyo III) was provided by Ciba-Geigy Ltd., Basle, Switzerland in 1976. This preparation was stated to contain 77% w/w calcitonin peptide and 12% acetic acid, chloride and water. This was distributed into ampoules coded 77/575 each containing approximately 7.7 μg of calcitonin peptide and 10 mg mannitol.

A preparation of human calcitonin synthesized by a solid phase procedure (lot No. K692–229–2) was provided by Armour Pharmaceutical Co., Kankakee, USA, in 1976. The nominal biological activity was 116 MRC units per mg. This was distributed into ampoules coded 77/574 each containing approximately 10 μg of calcitonin peptide and 10 mg mannitol.

Design of the study

Seven laboratories in 6 countries took part in the study, and the names of the participants are listed in the Annex. Throughout this paper each laboratory is identified by a code number which does not correspond to the order of listing.

Participants carried out assays using their normal bioassay procedure, and using freshly opened ampoules for each assay. Except in Laboratory 5 (two preparations in each assay), each assay included the three preparations issued. Laboratories 1 and 2 also included their house standards. Preparations were assayed at three dose levels with a minimum of 5 animals for each dose level, so that within-assay variation could be determined, and the assumptions of linearity and parallelism of the log dose-response lines could be tested.

Bioassay methods

All participants used conventional in vivo bioassay methods which depend on the acute hypocalcaemia induced in fasted young rats 1 h after intravenous (Kumar et al. 1965) or subcutaneous (Hirsch et al. 1964; Sturtridge & Kumar 1968) injection. Of the 7 participating laboratories, 2 provided data from subcutaneous assays, 4 from intravenous assays, and one used both bioassay methods. Details of variations in methods are given in Table 1. In all systems the doses of calcitonin were within the range 2–20 mU/100 g body weight.

Statistical analysis

Each assay was analysed as a multiple parallel line assay (Finney 1978), and the assumptions of linearity and parallelism were examined using analysis of variance. Assays showing significant departures from parallelism at the 5% level were rejected. Assays for which one or more preparations showed departures from linearity were plotted and examined in detail. If the curvature affected only one preparation and the estimates of slope did not differ significantly within the assay and agreed with those from other assays by the same laboratory, the assay was not rejected. Assays for which the departure from linearity was significant at the 5% level for more than one preparation were examined, and if the curvature was not consistent for all preparations these assays were rejected.

For each valid assay, an estimate of potency was computed together with a weight based on the reciprocal of the variance of the log potency.

A chi square was computed to test estimates of potency for homogeneity within each laboratory and when homogenous, estimates were combined to give a weighted geometric mean. Mean estimates for each laboratory were similarly tested for homogeneity between laboratories and combined. When the chi square was significant (P < 0.05) the set of estimates was examined in detail in an attempt to determine the source of heterogeneity.

Results

Tests of validity and comparison of the International Reference Preparation with other calcitonin preparations.

Statistical analysis of the assays led to the rejection of one assay from each of Laboratories 4 and 6 for non-parallelism and one assay from each of Laboratories 1 and 2 and two assays from Laboratory 4 for curvature of log dose-response lines. The potency estimates from each assay are shown in Fig. 1, and laboratory mean potency estimates and weights are given in Table 2.

Within each laboratory and also between laboratories, potency estimates for 77/575 in terms of the IRP were homogenous with an overall weighted geometric mean of 1.21 (95% confidence interval 1.02–1.27) IU/ampoule.

Potency estimates for 77/574 in terms of the IRP were somewhat more variable. Within each laboratory except Laboratory 2 estimates of potency
were homogenous. In Laboratory 2 the chi square for homogeneity was significant at the 1% level due to the inclusion of the largest of the three estimates which was consequently omitted. The chi square among all the laboratory mean estimates was also significant at the 1% level. On examination this was found to be due to estimates from Laboratories 1 and 4, at opposite extremes of the distribution and having large weights. The weight for the estimate from Laboratory 4 in particular was large, and hence was reduced by half so that the contribution would be equivalent to about 3 or 4 assays of this type (the same number as contributed by most other laboratories). This gave a chi square with 0.05 > P > 0.025. With the weight for the mean from Laboratory 4 reduced as shown in Table 2, the overall weighted geometric mean for potency estimates for 77/574 was 0.848 (95% confidence interval 0.805–0.894) IU/ampoule.

Comparison of assays using intravenous injections with assays using subcutaneous injections did not reveal any difference in results (Fig. 1).

Laboratories 1 and 2 each included a local house standard in their assays. Both working standards were preparations of synthetic human calcitonin from different sources and gave linear log dose-response curves which were parallel to those of the IRP in these assays. In Laboratory 1, 0.84 units of local standard (previously calibrated by bioassay using ampoules coded 70/234, now the IRP) was found to be equivalent to 1 IU of 70/234 (95% confidence interval 0.71–0.99 units/IU). In Laboratory 2, 5.24 μg of the local standard was found to be equivalent to 1 IU of 70/234 (95% confidence interval 4.29–6.41 μg/IU).

Laboratory 5 carried out one additional comparison of the IRP with the First International Reference Preparation of Salmon Calcitonin and two working standards, one of salmon calcitonin, the other of eel calcitonin. In this assay the slope of the log dose-response curve of the IRP was significantly different from the slopes obtained with the calcitonins from different animal species.

**Stability of the IRP**

Samples of the IRP stored in unopened ampoules at +20°C and +37°C for periods of up to 91 months were assayed in 2 laboratories against the IRP stored continuously at −20°C. No loss of biological activity was detected. Ampoules of the IRP stored at +20°C for 92 months had estimated potency (95% confidence interval) of 1.00 (0.77–1.30) relative to the IRP stored continuously at −20°C. A similar comparison for ampoules of the IRP stored at +37°C for 92 months gave a relative potency estimate (95% confidence interval) of 0.97 (0.75–1.26).

**Discussion**

The results of this study confirm that the preparation of human calcitonin in ampoules coded 70/234 is suitable to serve as an International Reference Preparation. The results of the compa-
Table 2.
Laboratory mean potency estimates of preparations of human calcitonin coded 77/574 and 77/575 expressed as IU per ampoule.

<table>
<thead>
<tr>
<th>Lab No.</th>
<th>Mean potency estimate</th>
<th>Number of assays</th>
<th>Combined weight</th>
<th>Mean potency estimate</th>
<th>Number of assays</th>
<th>Combined weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.72</td>
<td>3</td>
<td>1402</td>
<td>1.16</td>
<td>3</td>
<td>1455</td>
</tr>
<tr>
<td>2 1</td>
<td>0.75</td>
<td>2</td>
<td>482</td>
<td>1.07</td>
<td>3</td>
<td>666</td>
</tr>
<tr>
<td>3 2</td>
<td>0.84</td>
<td>3</td>
<td>1265</td>
<td>1.10</td>
<td>3</td>
<td>1274</td>
</tr>
<tr>
<td>4 1</td>
<td>0.90</td>
<td>4</td>
<td>1701</td>
<td>1.38</td>
<td>4</td>
<td>1621</td>
</tr>
<tr>
<td>4 2</td>
<td>0.96</td>
<td>7</td>
<td>3094 3</td>
<td>1.23</td>
<td>7</td>
<td>3042</td>
</tr>
<tr>
<td>5 4</td>
<td>0.82</td>
<td>3</td>
<td>450</td>
<td>1.11</td>
<td>3</td>
<td>758</td>
</tr>
<tr>
<td>6 2</td>
<td>0.97</td>
<td>3</td>
<td>392</td>
<td>1.47</td>
<td>3</td>
<td>379</td>
</tr>
<tr>
<td>7 1</td>
<td>0.76</td>
<td>2</td>
<td>202</td>
<td>1.24</td>
<td>2</td>
<td>203</td>
</tr>
<tr>
<td>Overall Mean</td>
<td>0.848</td>
<td>–</td>
<td>7567</td>
<td>1.213</td>
<td>–</td>
<td>9398</td>
</tr>
</tbody>
</table>

* In terms of IU defined by the IRP (code No. 70/234).
1 Assays using subcutaneous injection.
2 Assays using intravenous injection.
3 Reduced to 1547 for computation of overall mean.

Comparison of the IRP with salmon and eel calcitonsins emphasize the importance of using a standard of calcitonin of the appropriate species for potency estimations. In the biological assay systems considered here, the dose-response relationship obtained for the IRP was indistinguishable from that for two other synthetic human calcitonin preparations, 77/575 and 77/574, and in two laboratories was also indistinguishable from that for a further two synthetic preparations of human calcitonin used as house standards. Accelerated degradation studies showed that the IRP is stable. Moreover, the IRP (as the MRC Research Standard) has been widely used without reported problems. All participants agreed to recommend that the preparation of human calcitonin in ampoules coded 70/234 (previously the MRC Research Standard for human calcitonin) should be established as the first International Reference Preparation of Calcitonin, Human, for Bioassay. This proposal was accepted at the 30th meeting of the ECBS of WHO where the preparation was formally established. In order that there would be continuity of unitage, it was also agreed that 1.0 International Unit of Calcitonin, Human, for Bioassay, should be defined as the activity contained in the material in each ampoule of the IRP.

Annex

List of participants (in alphabetic order):
Dr. J. P. Aldred, Armour Pharmaceutical Co., Kankakee, Illinois, USA.
Dr. M. Azria, Laboratoires Sandoz, Rueil Malmaison, France.
Dr. D. M. Conning, Dr. D. Pelling, and Dr. K. R. Butterworth, British Industrial Biological Research Association,* Carshalton, UK.
Dr. R. Maier, Ciba-Geigy Ltd., Basle, Switzerland.
Dr. H. H. Petersen and Dr. H. L. Lembøl, The Danish National Health Service Pharmaceutical Laboratories, Brønshøj, Denmark.
Dr. T. Sakurada, Dr. K. Sugimoto, Dr. S. Watanabe and Dr. K. Hayano, Toyo Jozo Co. Ltd., Shizuoka, Japan.

Dr. J. M. Zanelli and Mr. B. Rafferty, National Institute for Biological Standards and Control, Hampstead, London, UK.

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References


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