Appendix 1a – Phase 1 study protocol

STUDY PROTOCOL

PROPOSED INTERNATIONAL COLLABORATIVE STUDY TO ESTABLISH THE 1st WHO INTERNATIONAL STANDARD FOR INSULIN, HUMAN – PHASE 1

INTRODUCTION

The current WHO standards for insulin (human – coded 83/500, bovine – coded 83/511, porcine – coded 83/515) were established in the mid 1980's. Their potencies are defined in IU/mg. The assignment of value to the current human insulin standard was based on a multi method collaborative study by *in vivo* bioassay, hence the need for an updated standard(s) to reflect the transition of insulin internationally to a well-characterized, mass-balance assigned molecule. With this in mind, a new preparation of insulin has been filled into ampoules (NIBSC code 11/212) following procedures recommended by WHO. It is now intended to set up an international collaborative study with expert laboratories to aid in the value assignment of the proposed IS.

The study will be conducted in **three phases** as follows:

Phase 1 – **Assignment of insulin content to the candidate standard.**

The insulin content of the candidate standard (on an as is basis) will be assigned based on data generated for the insulin content of the bulk active pharmaceutical ingredient (API) used to fill the candidate standard and the processing data from the definitive fill of the material into ampoules. Participants will be asked to use a mass balance approach to determine the insulin (plus A21 desamido insulin) content of the bulk API and submit this data to NIBSC for central processing. This data will be used in conjunction with the data from the filling of the candidate standard (mass of bulk API filled into each ampoule and coefficient of variation for the weight of the filled ampoules) to assign the final content to the candidate standard. The uncertainty will be assigned to this final value based on the combined uncertainties of the mass balance and filling data. Confirmatory data on the insulin content of the candidate standard will be provided using HPLC (candidate standard vs mass-balanced bulk API).

Phase 2a – Assessment of the suitability of the candidate standard to serve as an International Standard for the calibration of diagnostic immunoassays.

The performance of the candidate standard in the insulin immunoassays that are in current use will be assessed in an international collaborative study. This will include clinical samples to allow an assessment of the likely commutability of the standard.

Phase 2b – Assessment of the suitability of the candidate standard to serve as an International Standard for the calibration of secondary reference preparations used to assign potency to therapeutic preparations of insulin.

Regional pharmacopoeias have established their own reference standards to be used in their respective monographs. To reduce the potential for differences in the relative assignments between different pharmacopoeias and also between batches of reference standard from the same agency, there is a requirement for a single global human insulin standard which can be used to calibrate regional/compendial standards using HPLC methods. This would improve consistency between these standards which would ideally result in consistent dosing to patients worldwide and more straight-forward quality control for global manufacturers.

MATERIAL HANDLING

Recombinant insulin is very hygroscopic so all containers should be kept tightly closed. Protect both API and the candidate standard materials from light and store at minus 20^{o}C. Allow vials and ampoules to reach room temperature before opening.

MATERIALS SUPPLIED FOR PHASE 1

A bulk preparation of highly purified, recombinant human insulin was generously donated to the WHO by a manufacturer of therapeutic insulin. The bulk preparation was provided as 100 g of crystalline insulin API (batch AM0H22101).

Bulk insulin API: Approximately 25 g of the bulk API was removed in a dry box and further distributed under controlled relative humidity (3%) into smaller vials containing, either 100, 200, 250, 300 or 500 mg bulk insulin API for the tests associated with the mass balance characterisation. Vials containing approximately 50 mg were also prepared for HPLC analysis of the bulk API.

Candidate insulin standard (coded 11/212): 55.028 g of the bulk API was removed in a dry box under controlled humidity, dissolved in ddH_2O and acidified with 0.2 M HCl until the solution was clear. The acidified solution was neutralised to pH 7.4 with 0.2 M NaOH and the final volume was made up gravimetrically to 5503 g to give a concentration of insulin API of 10 mg/g final solution. This solution was dispensed in 1 g aliquots into glass ampoules, lyophilised and sealed.

Additional materials: Vials of the EDQM reference material; insulin (porcine) CRS are provided for the system suitability tests for the HPLC methods.

VALUE ASSIGNMENT PROTOCOL

Bulk insulin API

Data generated from the mass balance assessment of the insulin content of the bulk insulin API will be used in conjunction with the data from the filling of the candidate standard to assign the final insulin content to the candidate standard. As such, a full characterisation of the bulk insulin API is critical for the accurate assignment of insulin content to the candidate standard. Data from the collaborative study participants will be

used in conjunction with manufacturer's data for the batch of bulk insulin API "as is" in the mass balance assessment as follows:

Mass balance

Insulin plus related impurities = 100% - [%LOD + %zinc + %salts (Na^+, Cl^-, $Acet^-$)]

Final insulin content can be corrected for related impurities, obtained in HPLC assay on the candidate standard.

An example calculation using approximate (fictional) data is given below and provides an indication as to the likely contribution of each component to the overall mass balance calculation:

Insulin plus related impurities = 100% - [7.54% (LOD) + 0.39% (Zn)+ 0.4% (salts)]

Insulin plus related impurities = 91.67% or 91.67 mg per 100 mg insulin API "as is"

Insulin content = 91.67 x 0.996 (related impurities of 0.4%)

- where related impurities are assessed by HPLC on the candidate standard

= 91.30 mg per 100 mg insulin API "as is"

Given the relatively small contribution of the data for the salt content to the overall mass balance calculation and the specialist nature of the tests for these substances, it is anticipated that a smaller number of laboratories may perform these tests. In addition, the test for Zinc and the orthogonal test of insulin content of the API based on the measurement of total Nitrogen (see below) may also be performed by a smaller number of laboratories.

Total Nitrogen analysis

Although the insulin content of the bulk API will be assigned on the basis of mass balance, as described above, participants will also be requested, where possible, to use an orthogonal method which will be used to provide confirmatory data. Given the MW of insulin (5807.58), the atomic weight of nitrogen (14.0067) and the number of nitrogen (N) atoms per human insulin molecule (65) it is also possible to determine the theoretical insulin (plus related substances) content from an analysis of total N as follows:

Theoretical insulin = [(65 x 14.0067)/5807.58] x 100% = 15.68%

Using an experimentally derived measurement of the N content of the bulk API (e.g 14.20 %), it is possible to calculate the insulin content of the bulk insulin API as follows:

% N (bulk API) / % N (theoretical) = 14.20 / 15.68% = 0.9056

= 90.56 mg human insulin plus related impurities/100 mg insulin API

Human insulin content = 90.56 x 0.996 (related impurities of 0.4%)

= 90.20 mg per 100 mg insulin API "as is"

Candidate standard (11/212)

Data generated from the mass balance assessment of the insulin content of the bulk insulin API (corrected for related impurities using data from HPLC on the candidate standard) will be used in conjunction with the data from the filling of the candidate standard to assign the final insulin content to the candidate standard. The insulin solution used to fill the candidate standard was prepared to give a concentration of insulin API "as is" of 10.00 mg / g final solution. This solution was dispensed in 1g aliquots into glass ampoules, lyophilised and sealed. Data from the fill was as follows:

Mean filling weight = 1.0002 g per ampoule (RSD of 0.12%)

Using the fictional data from the insulin API mass balance example above (91.30 mg per 100 mg insulin API "as is") and this mean filling weight, the insulin plus A21 desamido content of the candidate standard can be calculated as follows:

Insulin plus A21 desamido = 0.9130 x 10 x 1.0002

= 9.132 mg per ampoule

Uncertainty

Standard uncertainty on insulin content of candidate standard:

$$\sqrt{u_{LOD}^2+u_{Zn}^2+u_{salts}^2+u_{rel\ imp}^2+u_{fill\ wt}^2}$$

where u_{LOD}, u_{Zn}, u_{salts}, $u_{rel\ imp}$, denote standard uncertainties due to tests for loss on drying, zinc content, salt content and related impurities and $u_{fill\ wt}$ denotes the standard uncertainty for the filling weight.

TESTS REQUESTED

Bulk insulin API

Table 1: Tests for mass balance assessment of the bulk insulin API

Test	mg insulin API/test
Water content (EP 2.2.32 – LOD)	**200**
Zinc (EP 2.2.23 - Atomic absorption spectrometry - AAS)	**50**
Nitrogen (Elemental or Kjeldahl)	**200**
Sodium (EP 2.2.23 – AAS)	**250**
Chloride and Acetate (Ion chromatography)	**100**

NB The tests of insulin content and related impurities by HPLC (EP 2.2.29) are also requested on the bulk insulin API as part of the control check of the insulin content of the candidate standard (see later).

Water determination - Loss on drying (EP 2.2.32)

Handling of materials for water determination (see appendix 1): Vials containing 500 mg bulk insulin API are provided for the analysis of water content.

Participants are requested to follow the appropriate pharmacopeial methods where possible and to report their data using the data return sheet (table 3; see appendix 2). Where participants use an in-house method (e.g. Karl Fisher titration), they are requested to provide details on this sheet.

Zinc determination by atomic absorption spectroscopy (EP 2.2.23).

Handling of materials for Zinc determination: Approximately 200 mg bulk insulin API is provided for the analysis of zinc content.

Participants are requested to follow the appropriate pharmacopeial methods where possible (see appendix 4). Where participants use an in-house method, they are requested to provide details in their report.

Nitrogen determination by Elemental or Kjeldahl method

Handling of materials for nitrogen determination: Vials containing 200 mg bulk insulin API are provided for the analysis of nitrogen content.

Where participants indicate they are able to provide data for the determination of nitrogen content, an example protocol is available upon request. Participants are requested to provide details of the method used in their report.

Sodium determination by atomic absorption spectroscopy (EP 2.2.23).

Handling of materials for sodium determination: Approximately 250 mg bulk insulin API is provided for the analysis of sodium content.

Participants are requested to follow the appropriate pharmacopeial methods where possible (see appendix 4). Where participants use an in-house method, they are requested to provide details in their report.

Chloride and Acetate determination by Ion chromatography

Handling of materials for chloride and acetate determination: Approximately 100 mg bulk insulin API is provided for the each analysis of chloride or acetate content.

Where participants use an in-house method, they are requested to provide details in their report.

Candidate Standard (11/212)

Assay of insulin content and related impurities by HPLC – (EP 2.2.29)

The final insulin content assigned to the candidate standard, which is based on the mass balance and filling data, can be corrected for related impurities, using data obtained from HPLC assays for related impurities performed on the candidate standard.

In addition, as a secondary check of the insulin content of the candidate standard, HPLC assay of the candidate standard in terms of the bulk insulin API will be requested. This second set of data will not be used in the value assignment of the insulin content of the candidate standard.

Handling of materials (see appendix 1): Ampoules of the candidate standard, coded 11/212, containing 10 mg bulk insulin API (lyophilised) and vials containing 50 mg bulk insulin API are provided for the assays of insulin content and related peptides/impurities.

Participants are requested to follow the appropriate pharmacopeial methods and to report their data using the data return sheets provided (see appendix 3).

REPORT

A preliminary report will be prepared and circulated to all participants for comment before submission to the Expert Committee on Biological Standardization of WHO. In the report, participating laboratories will be identified by a laboratory number only and any requests to treat information in confidence will be respected. For further information, please contact:

Dr. Chris Burns, Principal Scientist, Endocrinology, Biotherapeutics, NIBSC

Tel: 01707 641247; Email: Chris.Burns@nibsc.hpa.org.uk

APPENDIX 1: WEIGHING SAMPLES.

Crystalline insulin is very hygroscopic and so care must be taken when weighing out the samples for the proposed tests to characterise the insulin API.

Table 2: insulin provided for the tests to characterise the bulk insulin API

Test on Insulin API	mg insulin API provided
Water content (EP 2.2.32 – LOD)	**3 x 500**
Zinc (EP 2.2.23 - Atomic absorption spectrometry - AAS)	**1 x 200**
Nitrogen (Kjeldahl)	**6 x 200**
Sodium (EP 2.2.23 – AAS)	**3 x 250**
Chloride and Acetate (Ion chromatography)	**6 x 100**
Assay of insulin content and related impurities (EP 2.2.29 - HPLC)	**6 x 50**

The relative humidity (RH) of the laboratory where the weighing is taking place should ideally be 20-40%. Please report the RH of the laboratory during weighing.

Samples should be weighed quickly and accurately. An example of the weighing protocol for the test to determine water content (LOD) of the insulin API is descibed in appendix 2 below but can be broadly applied to all tests:

APPENDIX 2: DETERMINING WATER CONTENT OF INSULIN API.

Loss on drying (LOD) is the loss of mass expressed as % m/m upon drying. Care should be taken when weighing the samples.

- Duplicate measurements for LOD should be made from each vial on the same day and approximately the same amount of time should be taken to weigh out each sample for the test (200 mg). Use the same balance for each weighing and report the time taken to perfrom all weighings. Every effort should be made to minimise the time that the stopper is off the vial.
- Allow one vial containing 500 mg insulin API to reach room temperature.
- In duplicate, weigh approximately 200 mg insulin API accurately to 2 decimal places in a pre-dried weighing vessel, recap the vial immediately and perform water determination by drying the insulin to constant mass in an oven at 105±2^{o}C for 24 hours. Report the results in **Table 3**.
- Repeat all steps on the following day with a second vial.
- Repeat all steps on the third day with the third vial

Data reporting sheet

Laboratory: **Date:**

RH of laboratory:

Total time taken for all weighings:

Table 3: LOD of human insulin bulk API

	Vial 1 Day 1		Vial 2 Day 2		Vial 3 Day 3	
	1	2	1	2	1	2
Weight of weighing vessel						
Weight of vessel + insulin						
Weight of vessel +dried insulin						
Loss on drying						
Mean LOD for vial						
Mean (n=3)						
Standard deviation						

Additional Information:

APPENDIX 3: HPLC ASSAY OF INSULIN CONTENT AND RELATED PEPTIDES/IMPURITIES

Maintain the solutions at 2-10 °C and use within 24 h.

Candidate standard test solution. Dissolve the contents of an ampoule of the candidate standard, coded 11/212, in 2.25ml 0.01 M hydrochloric acid. Record the mass of the 2.25 ml hydrochloric acid added, on the data sheet provided.

Reference solution (a). Dissolve the contents of a vial of insulin (porcine) CRS in 0.01M hydrochloric acid to obtain a concentration of 4.0 mg/mL.

Reference solution (b). Exactly weigh about 40.0 mg of human insulin bulk, dissolve in 0.01 M hydrochloric acid and complete the volume to 10.0 mL in a volumetric flask with the same solvent. Record the total mass of the hydrochloric acid added, on the data sheet provided.

Reference solution (c). Dilute 1.0 mL of reference solution (b) to 10.0 mL with 0.01 M hydrochloric acid.

Resolution solution . Mix 1.0 mL of reference solution (b) and 1.0 mL of reference solution (a).

Column :
— size :l = 0.25 m, Ø = 4.6 mm,
— stationary phase :octadecylsilyl silica gel for chromatography R (5 μm),
— temperature : 40 °C

System suitability:
— *resolution* : inject 20 μL of the resolution solution and 20 μL of reference solution (a). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (a) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks corresponding to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.

linearity : inject 20 μL each of reference solutions (c) and (b). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (b) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c). If this test fails, adjust the injection volume to between 10 μL and 20 μL, in order that the responses are within the linearity range of the detector.

Report results of system suitability tests in **Table 4**.

<u>Data reporting sheet</u> : System suitability tests for HPLC.

Laboratory: **Date:**

Report any adjustment of mobile phase, gradient or injection volumes:

Table 4: System suitability tests for HPLC.

	Limits	Injection 1	Injection 2	Injection 3
Resolution between human and porcine insulin peaks	≥1.2			
Linearity of detector (reference b vs reference c)	10 ± 0.5 times			

Please copy this table if required

3A: HPLC ASSAY OF RELATED PEPTIDES/IMPURITIES

mobile phase A : dissolve 28.4 g of anhydrous sodium sulphate R in water R and dilute to 1000 ml with the same solvent; add 2.7 mL of phosphoric acid R ; adjust to pH 2.3, if necessary, with ethanolamine R ; filter and degas

mobile phase B : mix 550 mL of mobile phase A with 450 ml of acetonitrile R. Warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

Flow rate: 1 mL/min.

Detection: spectrophotometer at 214 nm.

Mobile phases as above using the following gradient:

Table 5: Gradient for test for related peptides/impurities of the bulk insulin API.

Time (minutes)	Mobile phase A (% v/v)	Mobile phase B (%v/v)
0-30	42	58
30-44	42→11	58→89
44-50	11	89

System suitability: (resolution, linearity) as described above. If necessary, the relative proportions of the mobile phases may be adjusted to ensure complete elution of A21 desamido porcine insulin before commencement of the gradient. The profile of the gradient may also be adjusted to ensure complete elution of all insulin related impurities.

Perform the tests for related peptides/impurities on both the bulk insulin API and candidate standard to allow for the correction of insulin content of the candidate standard and also to determine the effect of lyophilisation on insulin purity. Perform three (3) independent tests, using i) a separate ampoule of the candidate standard or ii) a separate weighing from a new vial of the bulk to produce reference solution (b), for each test. Inject 20 µL of this solution three (3) times. If necessary, adjust the injection volume to between 10 µL and 20 µL in accordance with the results obtained in the test for linearity as described under Assay. Record the chromatograms for approximately 50 min. In the chromatogram obtained with reference solution (b), A21 desamido human insulin appears as a small peak eluting after the principal peak and has a relative retention of about 1.3 with reference to the principal peak. **Do not report the A21 desamido human insulin as a related impurity.** Using the chromatograms obtained from each of the three independent tests described above, calculate by normalisation for each injection the related protein content of the human insulin bulk API and the candidate standard.

Report all the results in **Table 6**. Attach the corresponding chromatograms.

<u>Data reporting sheet</u> : Related peptides/impurities (please copy as appropriate)

Candidate standard ☐ **(Tick as appropriate)**

Bulk insulin API ☐

Laboratory: **Date:**

HPLC equipment and detector:

Column details (column dimensions and particle size):

Table 6: HPLC assay of related peptides/impurities

			Calculated percentage by normalisation		
Peak Identification number	**Retention time (min)**	**Relative retention***	**Injection 1**	**Injection 2**	**Injection 3**
Sum of impurities					

Please copy this table and indicate either i) insulin bulk API or ii) candidate standard and whether test 1, 2 or 3

*Relative to the main human insulin peak

3B: HPLC ASSAY - INSULIN CONTENT OF CANDIDATE STANDARD.

Column and mobile phases A and B as above.

Mobile phase: mix 42 volumes of mobile phase A and 58 volumes of mobile phase B, adjusting the composition of the mixture if necessary.

Flow rate: 1 mL/min.

Detection: spectrophotometer at 214 nm.

System suitability: (resolution, linearity) as described above (page 9).

Perform three (3) independent assays, using a new ampoule of the candidate standard (test) and a new weighing of the bulk (reference solution b) for each of the assays. Use reference solution (b) and the test solution, injecting 20 µL of each solution three (3) times. If necessary, adjust the injection volume to between 10 µL and 20 µL in accordance with the results obtained in the test for linearity. Report the areas of the human insulin and A21 desamido human insulin peaks in **Table 7**. Calculate the means and RSD of the human insulin peak areas. The RSD must be less than 1.04 %. Calculate the content of human insulin plus A21 desamido human insulin and report in **Table 7**.

Attach the corresponding chromatograms.

Data reporting sheet (please copy as appropriate)

Laboratory: **Date:**

HPLC equipment and detector:

Column details (column dimensions and particle size):

Mass of HCl added to ampoule of candidate standard (mg):

Total mass of HCl added to bulk insulin to make Reference solution b (mg):

Table 7: HPLC assay - insulin content of candidate standard.

	Injection				
Test Solution (Candidate Standard)	**1**	**2**	**3**	**Mean**	**RSD (≤1.04%)**
Human insulin peak area					
A21 desamido peak area					
Sum of human insulin + A21 desamido peak area means = A					
	Injection				
Reference Solution (b)	**1**	**2**	**3**	**Mean**	**RSD (≤1.04%)**
Human insulin peak area					
A21 desamido peak area					
Sum of human insulin + A21 desamido peak area means = B					
Mass of bulk insulin to make reference solution (b)				**mg**	

Content of human insulin plus A21 desamido insulin per ampoule of the candidate standard is given by:

$$\frac{\text{A x mass (mg) of HCl added to ampoule}}{\text{B x mass (mg) HCl added to ref (b)/mass bulk insulin in ref (b)}} \text{ x insulin content of bulk material (mg /mg "as is")}$$

APPENDIX 4: DETERMING ZINC CONTENT OF THE INSULIN API

Follow EP 2.2.23. Atomic absorption spectrometry (method I – direct calibration)

Test solution: Dissolve 50.0 mg of the substance to be examined in *0.01 M hydrochloric acid* and dilute to 25.0 ml with the same acid. Dilute if necessary to a suitable concentration (for example, 0.4-1.6 µg of Zn per millilitre) with *0.01 M hydrochloric acid.*

Reference solutions: Use solutions containing 0.40 µg, 0.80 µg, 1.00 µg, 1.20 µg and 1.60 µg of Zn per millilitre, freshly prepared by diluting *zinc standard solution (5mg/ mL Zn) R* with *0.01 M hydrochloric acid.*

Source: Zinc hollow-cathode lamp.

Wavelength: 213.9nm.

Atomisation device: air-acetylene flame of suitable composition (for example, 11 L or air and 2 L of acetylene per minute).

Appendix 1b – Phase 1 repeat study protocol

STUDY PROTOCOL

PROPOSED INTERNATIONAL COLLABORATIVE STUDY TO ESTABLISH THE 1st WHO INTERNATIONAL STANDARD FOR INSULIN, HUMAN

PHASE 1 Repeat

INTRODUCTION

The current WHO standards for insulin (human – coded 83/500, bovine – coded 83/511, porcine – coded 83/515) were established in the mid-1980s. Their potencies are defined in IU/mg. The assignment of value to the current human insulin standard was based on a multi method collaborative study by *in vivo* bioassay, hence the need for an updated standard(s) to reflect the transition of insulin internationally to a well-characterized, mass-balance assigned molecule. With this in mind, a new preparation of insulin has been filled into ampoules (NIBSC code 11/212) following procedures recommended by WHO and an international collaborative study was organised with expert laboratories to aid in the value assignment of the proposed IS.

Following the receipt and analysis of data from Phase I, it became apparent that estimates of the insulin content of the candidate standard by HPLC assay were lower than the expected content determined by mass balance.

With the support of one of the study participants, an investigation of the discrepancy has suggested that the procedure to weigh the bulk API into smaller vials (sub-portions) for the mass balance tests may have inadvertently caused drying of the material. The loss of water from the API sub-portions is likely to have had an impact on the measurements of loss on drying, nitrogen determination and the HPLC assay of insulin content. This drying process did not occur in the handling of the material used to prepare the candidate standard and this inconsistency is likely to have caused the observed discrepancy in insulin content. The aim of the Phase I Repeat study is to repeat these procedures using freshly sub-portioned bulk API to obtain an accurate estimate of the insulin content of the bulk API and the insulin content of the candidate standard by HPLC assay.

As described in the original study protocol, the study to establish the WHO International standard (IS) for insulin, human will be conducted in **three phases** as follows:

Phase 1 – **Assignment of insulin content to the candidate standard.**

The insulin content of the candidate standard (on an "as is" basis) will be assigned based on data generated for the insulin content of the bulk active pharmaceutical ingredient (API) used to fill the candidate standard and the processing data from the definitive fill of the material into ampoules. Participants will be asked to use a mass balance approach to determine the insulin (plus A21 desamido insulin) content of the bulk API and submit this data to NIBSC for central processing. This data will be used in conjunction with the data from the filling of the candidate standard (mass of bulk API filled into each ampoule and coefficient of variation for the weight of the filled ampoules) to assign the final content to the candidate standard. The uncertainty will be assigned to this final value based on the combined uncertainties of the mass balance and filling data. Confirmatory data on the insulin content of the candidate standard will be provided using HPLC (candidate standard vs mass-balanced bulk API).

Phase 2a – Assessment of the suitability of the candidate standard to serve as an International Standard for the calibration of diagnostic immunoassays.

The performance of the candidate standard in the insulin immunoassays that are in current use will be assessed in an international collaborative study. This will include clinical samples to allow an assessment of the likely commutability of the standard.

Phase 2b – Assessment of the suitability of the candidate standard to serve as an International Standard for the calibration of secondary reference preparations used to assign potency to therapeutic preparations of insulin.

Regional pharmacopoeias have established their own reference standards to be used in their respective monographs. To reduce the potential for differences in the relative assignments between different pharmacopoeias and also between batches of reference standard from the same agency, there is a requirement for a single global human insulin standard which can be used to calibrate regional/compendial standards using HPLC methods. This would improve consistency between these standards which would ideally result in consistent dosing to patients worldwide and more straight-forward quality control for global manufacturers.

MATERIAL HANDLING

Recombinant insulin is very hygroscopic so all containers should be kept tightly closed. Protect both API and the candidate standard materials from light and store at minus 20°C. Allow vials and ampoules to reach room temperature, protected from light, before opening.

MATERIALS SUPPLIED FOR PHASE 1 Repeat

A bulk preparation of highly purified, recombinant human insulin was generously donated to the WHO by a manufacturer of therapeutic insulin. The bulk preparation was provided as 100 g of crystalline insulin API (batch AM0H22101).

Bulk insulin API: Approximately 25 g of the bulk API was distributed under ambient conditions into smaller vials containing, either 100, 200, 250, 300 or 500 mg bulk insulin API for the tests associated with the mass balance characterisation. Vials containing approximately 50 mg were also prepared for HPLC analysis of the bulk API.

Candidate insulin standard (coded 11/212): 55.028 g of the bulk API was dissolved in ddH_2O and acidified with 0.2 M HCl until the solution was clear. The acidified solution was neutralised to pH 7.4 with 0.2 M NaOH and the final volume was made up gravimetrically to 5503 g to give a concentration of insulin API of 10 mg/g final solution. This solution was dispensed in 1g aliquots into glass ampoules, lyophilised and sealed.

Additional materials: If required, participants will be provided with ampoules of the WHO International Standard for Insulin, Porcine, coded 83/515, for the system suitability tests for the HPLC methods.

SUMMARY OF THE ORIGINAL DATA OBTAINED FROM PHASE 1

Data generated from the mass balance assessment of the insulin content of the bulk insulin API was used in conjunction with the data from the filling of the candidate standard to assign the final insulin content to the candidate standard.

Bulk insulin API

Data from the collaborative study participants was used in conjunction with manufacturer's data for the batch of bulk insulin API "as is" in the mass balance assessment as follows:

The insulin content (plus related impurities) of the bulk insulin API can be derived from the following equation:

Insulin plus related impurities = 100% - [%LOD + %zinc + %salts (Na^+, Cl^-, $Acet^-$)]

The final insulin content can be corrected for related impurities, obtained in HPLC assay on the candidate standard. Actual data obtained from phase 1 is shown in Appendix 4 and is summarised below:

Insulin plus related impurities = 100% - [6.9095% (LOD) + 0.363% (Zn)+ 0.423% (salts)]

Insulin plus related impurities = 92.30% or 92.30 mg per 100 mg insulin API "as is"

Insulin content = 92.30 x 0.9969 (related impurities of 0.3130%)

- where related impurities are assessed by HPLC on the candidate standard

= 92.016 mg per 100 mg insulin API "as is"

Total Nitrogen analysis

Although the insulin content of the bulk API will be assigned on the basis of mass balance, as described above, participants in phase 1 were also requested, where possible, to use an orthogonal method which was used to provide confirmatory data. Given the MW of insulin (5807.58), the atomic weight of nitrogen (14.0067) and the number of nitrogen (N) atoms per human insulin molecule (65) it is possible to determine the theoretical insulin (plus related substances) content from an analysis of total N as follows:

Theoretical insulin = [(65 x 14.0067)/5807.58] x 100% = 15.68%

Using the experimentally derived data from phase 1 for the N content of the bulk API (see Appendix 4), it was also therefore possible to calculate the insulin content of the bulk insulin API as follows:

% N (bulk API) / % N (theoretical) = 14.50% / 15.68% = 0.9247

= 92.47 mg human insulin plus related impurities/100 mg insulin API

Human insulin content = 92.47 x 0.9969 (related impurities of 0.3130%)

= 92.187 mg per 100 mg insulin API "as is"

This was in good agreement with the estimate based on the mass balance data.

Candidate standard (11/212)

Data generated from the mass balance assessment of the insulin content of the bulk insulin API (corrected for related impurities using data from HPLC on the candidate standard) will be used in conjunction with the data from the filling of the candidate standard to assign the final insulin content to the candidate standard. The insulin solution used to fill the candidate standard was prepared to give a concentration of insulin API "as is" of 10.00 mg/g final solution. This solution was dispensed in 1g aliquots into glass ampoules, lyophilised and sealed. Data from the actual fill of 11/212 was as follows:

Mean filling weight = 1.0002 g per ampoule (RSD of 0.12%)

Using actual data from the insulin API mass balance above (92.016 mg per 100 mg insulin API "as is") and this mean filling weight, the insulin plus A21 desamido content of the candidate standard can be calculated as follows:

Insulin plus A21 desamido = 0.92016 x 10 x 1.0002

= 9.203 mg per ampoule

An important check of the content assigned to the candidate standard by mass balance can be performed by assaying the candidate standard against the bulk material by HPLC. Appendix 4 shows the data from phase 1 for the content of the candidate standard derived from the assay of the candidate standard against the bulk material (Table 3). This data gives a mean estimate for the insulin content of the candidate standard of 9.059 mg per ampoule. This is some 1.6% lower than that obtained from the mass balance value-assignment (9.203 mg per ampoule).

Initial investigations into this discrepancy showed that the insulin content was homogenous (0.1% RSD) across the fill of the candidate standard. Although the possibility remains that a small amount of material may have been lost during the process of lyophilisation of the candidate standard, we hypothesised that our handling of the materials during processing may have led to the observed discrepancy in insulin content.

Briefly, the sub-portioning of bulk insulin API into containers for the mass balance tests (and also for the HPLC assay) took place under conditions of low relative humidity, in a dry box, using a portion of the bulk insulin which had been separated from that to be used in the fill. It is therefore possible that water was lost during the sub-portioning

process resulting in the sub-portioned insulin containing a different amount of insulin per milligram of weighed material than the bulk insulin used in the fill. Thus, if drying had indeed taken place, we would anticipate that the sub-portions would contain less water, and therefore more insulin, per milligram of weighed material than the stored bulk insulin API, offering a possible explanation for the observed discrepancy.

In order to test this hypothesis and to determine whether a repeat of the phase 1 study was warranted, the content of the remaining sub-portions from the original study were assayed by HPLC. The insulin content of a portion of the stored bulk insulin that had not been exposed to low relative humidity was also assayed by HPLC. We anticipated that the sub-portions from phase 1 would have higher insulin content per unit mass than the stored bulk because they had lost water during processing. Indeed, this was observed and the mean difference in insulin content was approximately 1.8%.

Confirmatory data were then sought from the donating manufacturer. Briefly, this laboratory was asked to perform the mass balance tests on newly prepared sub-portions of the bulk insulin that had been weighed out under conditions that were similar to the conditions used to weigh the bulk insulin for the candidate standard (i.e. 20-25^{o}C and 20-40% relative humidity). The LOD results obtained for these "non-dried" insulin sub-portions strongly suggested that they contain more water than the sub-portions that were previously measured by this participant in the original phase 1 study (7.39% vs 7.06% ($P<0.05$)). When the new LOD and mass balance data from this participant were used in the mass balance calculation, the estimated content of the candidate standard was 9.144 mg per ampoule.

The laboratory was also asked to derive new data from the HPLC assay of the insulin content of the ampoules of candidate standard in terms of the new "non-dried" bulk. This generated a mean estimate for the insulin content of the candidate standard (9.047 mg per ampoule) that, in this lab, was much closer to the value derived from the mass balance data (1.06% difference) in comparison to the difference observed by the same laboratory in the original phase 1 study (2.21%).

These data, when taken together, suggest that a significant component of the discrepancy in estimates of insulin content observed in the original phase 1 study is likely to be as a result of "drying" of the sub-portions that were prepared for the individual tests requested. As a result, we would like to revisit this part of the study using new bulk material and we are now asking participants to perform a small number of additional tests. The data from the original study, supported by the second set of analyses from the donating manufacturer, also demonstrated that the values derived for the content of the non-insulin components of the bulk material, namely zinc, sodium,

acetate and chloride were consistent between the two studies and were in agreement with laboratories in phase 1. Given that these contribute relatively little to the mass balance calculation when compared to the LOD, we will not ask for a repeat of these measurements and will use the original data from phase 1.

TESTS REQUESTED FOR PHASE 1 REPEAT

Bulk insulin API

Table 1: Tests on bulk insulin API for Phase 1 Repeat

Test	Insulin API/test (mg)
Water content (EP 2.2.32 – LOD)	**200**
Nitrogen (Elemental or Kjeldahl)	**200**

NB The assay of insulin content by HPLC (EP 2.2.29) is also requested and will require measurement of the bulk insulin API as part of the control check of the insulin content of the candidate standard (see later).

Water determination - Loss on drying (EP 2.2.32)

Handling of materials for water determination (see Appendix 1): Vials containing approximately 500 mg bulk insulin API are provided for the analysis of water content.

Participants are requested to follow the appropriate pharmacopeial methods where possible and to report their data using the data return sheet (table 3; see Appendix 2). Where participants use an in-house method (e.g. Karl Fisher titration), they are requested to provide details on this sheet.

Nitrogen determination by Elemental or Kjeldahl method

Handling of materials for nitrogen determination: Vials containing approximately 200 mg bulk insulin API are provided for the analysis of nitrogen content.

Where participants indicate they are able to provide data for the determination of nitrogen content, an example protocol is available upon request. Participants are requested to provide details of the method used in their report.

Candidate Standard (11/212)

Assay of insulin content by HPLC (EP 2.2.29)

The final insulin content assigned to the candidate standard, which is based on the mass balance and filling data, can be corrected for related impurities, using data obtained from HPLC assays for related impurities performed on the candidate standard in Phase I.

As part of Phase I Repeat, the insulin content of the candidate standard as determined by HPLC assay of the candidate standard in terms of the bulk insulin API will be requested. As described previously, this second set of data will not be used in the value assignment of the insulin content of the candidate standard.

Handling of materials for HPLC Assay of insulin content (see Appendices 1 and 3): Ampoules of the candidate standard, coded 11/212, containing 10 mg bulk insulin API (lyophilised) and vials containing approximately 50 mg bulk insulin API are provided for the assays of insulin content.

Participants are requested to follow the appropriate pharmacopeial methods and to report their data using the data return sheets provided (see Appendix 3).

REPORT

A preliminary report will be prepared and circulated to all participants for comment before submission to the Expert Committee on Biological Standardization of WHO. In the report, participating laboratories will be identified by a laboratory number only and any requests to treat information in confidence will be respected. For further information, please contact:

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APPENDIX 1: WEIGHING SAMPLES

Crystalline insulin is very hygroscopic and so care must be taken when weighing out the samples for the proposed tests to characterise the insulin API.

Table 2: insulin provided for the tests to characterise the bulk insulin API

Test on Insulin API	Insulin API provided (mg)
Water content (EP 2.2.32 – LOD)	**3 x 500**
Nitrogen (Kjeldahl)	**6 x 200**
Assay of insulin content (EP 2.2.29 - HPLC)	**4 x 50**

The relative humidity (RH) of the laboratory where the weighing is taking place should ideally be 20-40%. Please report the RH of the laboratory during weighing.

Samples should be weighed quickly and accurately. An example of the weighing protocol for the test to determine water content (LOD) of the insulin API is descibed in Appendix 2 but can be broadly applied to other mass balancetests. Instructions for the weighing of insulin API for use in the HPLC assay are given in Appendix 3

APPENDIX 2: DETERMINING WATER CONTENT OF INSULIN API.

Loss on drying (LOD) is the loss of mass expressed as % m/m upon drying. Care should be taken when weighing the samples.

- Duplicate measurements for LOD should be made from each vial on the same day and approximately the same amount of time should be taken to weigh out each sample for the test (200 mg). Use the same balance for each weighing and report the time taken to perform all weighings. Every effort should be made to minimise the time that the stopper is off the vial.
- Allow one vial containing 500 mg insulin API to reach room temperature.
- In duplicate, weigh approximately 200 mg insulin API accurately to 2 decimal places in a pre-dried weighing vessel, recap the vial immediately and perform water determination by drying the insulin to constant mass in an oven at 105 ± 2^{o}C for 24 hours. Report the results in **Table 3**.
- Repeat all steps on the following day with a second vial.
- Repeat all steps on the third day with the third vial

Data reporting sheet

Laboratory: **Date:**

RH of laboratory:

Total time taken for all weighings:

Table 3: LOD of human insulin bulk API

	Vial 1 Day 1		**Vial 2 Day 2**		**Vial 3 Day 3**	
	1	**2**	**1**	**2**	**1**	**2**
Weight of weighing vessel						
Weight of vessel + insulin						
Weight of vessel + dried insulin						
Loss on drying						
Mean LOD for vial						
Mean (n=3)						
Standard deviation						

Additional Information:

APPENDIX 3: HPLC ASSAY OF INSULIN CONTENT

Preparation of test and reference solutions

Maintain the solutions at 2-10 °C and use within 24 h.

Candidate standard test solution: Allow an ampoule of the candidate standard, 11/212, to reach room temperature (20-25°C) for 1h and protected from light, before opening. Ensure all the ampoule contents are in the body of the ampoule and open the ampoule using the protective sleeve provided. Using a calibrated balance reading to 4 decimal places, weigh the opened ampoule and dissolve the contents in a 2.25 mL volume of 0.01 M hydrochloric acid. Record the exact mass of the hydrochloric acid added on the data sheet provided (**Table 5**). Seal the opened vial with parafilm, mix gently and leave on ice for 30 min protected from light. Inspect the ampoule and ensure that all the ampoule contents are dissolved.

For each independent HPLC assay, prepare one candidate standard test solution from a

previously unopened ampoule of the candidate standard, 11/212.

Reference solution (a): Dissolve the contents of a vial of insulin (porcine) CRS or one ampoule of WHO International Standard for porcine insulin (coded 83/515) in 0.01M hydrochloric acid to obtain a concentration of 4.0 mg/mL.

Reference solution (b): Allow a vial containing 50 mg of human insulin bulk to reach room temperature (20-25°C), for 1h and protected from light, before opening. Turn and rotate the sample container gently a few times before opening to ensure that the sample is homogeneous. Gently tap the bottom of the container against the table a few times to ensure that the sample is at the bottom of the container before opening.

When weighing material, open the vial for the minimum time, if possible at 20-40% relative humidity and close the container between transfers of material. Using a calibrated balance reading to 4 decimal places, accurately weigh about 40.0 mg of human insulin bulk into a sealable, single use, glass vessel (For example: 15 mL clear vial, screw top, solid cap with PTFE liner; Sigma-Aldrich, Cat No 27161).

In **Table 5**, record the mass of human insulin bulk weighed, which should be between 38 and 42 mg. Add a 10 mL volume of pre-chilled, 0.01 M hydrochloric acid, seal, and record the mass of hydrochloric acid added on the data sheet provided (**Table 5**). Rotate gently, protected from light at 4°C for 30 minutes. Inspect to ensure that all the human

insulin bulk has been dissolved and there is no material remaining on the neck or sides of the 15mL vial. If the insulin is not dissolved, continue to rotate until it is dissolved.

For each independent HPLC assay, prepare one solution of reference solution (b) from a previously unopened vial of human insulin bulk.

Reference solution (c): Dilute 1.0 mL of reference solution (b) to 10.0 mL with 0.01 M hydrochloric acid.

Resolution solution: Mix 1.0 mL of reference solution (b) and 1.0 mL of reference solution (a).

Chromatography conditions

Column:

— size: l = 0.25 m, Ø = 4.6 mm,

— stationary phase: octadecylsilyl (C18) silica gel for chromatography R (5 μm)

— temperature: 40 °C

Mobile phase A: dissolve 28.4 g of anhydrous sodium sulphate R in water R and dilute to
1000 mL with the same solvent; add 2.7 mL of phosphoric acid R; adjust to pH 2.3, if necessary, with ethanolamine R; filter and degas.

Mobile phase B: mix 550 mL of mobile phase A with 450 mL of acetonitrile R. Warm the solution to a temperature of at least 20 °C in order to avoid precipitation (mixing of mobile phase A with acetonitrile is endothermic); filter and degas.

Mobile phase for HPLC Assay of Insulin: Isocratic elution using 42% mobile phase A, 58% mobile phase B. The mobile phase conditions for the HPLC assay should be adjusted to ensure (i) the system suitability criteria for resolution is met and (ii) the desamido insulin peak of reference solution (a) elutes within 30 min.

Flow rate: 1 mL/min.

Detection: spectrophotometer at 214 nm.

System suitability:

Resolution: Inject 20 µL of the resolution solution and 20 µL of reference solution (a). Record the chromatogram of the resolution solution until the peak corresponding to the principal peak in the chromatogram obtained with reference solution (a) is clearly visible. In the chromatogram obtained with the resolution solution, identify the peaks due to porcine insulin and human insulin. The test is not valid unless the resolution between the peaks corresponding to human insulin and porcine insulin is at least 1.2. If necessary, adjust the concentration of acetonitrile in the mobile phase until this resolution is achieved.

Linearity: Inject 20 µL each of reference solutions (c) and (b). The test is not valid unless the area of the principal peak in the chromatogram obtained with reference solution (b) is 10 ± 0.5 times the area of the principal peak in the chromatogram obtained with reference solution (c). If this test fails, adjust the injection volume to between 10 µL and 20 µL, in order that the responses are within the linearity range of the detector.

Relative standard deviation: Inject 20 µL of reference solution (b) in triplicate. Calculate the mean and RSD of the human insulin peak areas. The RSD should be less than 1%.

Report the results of the system suitability tests in **Table 4**.

HPLC Assay

Perform three (3) independent assays, using a new ampoule of the candidate standard, 11/212, for the preparation of the test solution and a new vial containing 50 mg of the insulin bulk for the preparation of reference solution (b) for each of the assays.

Use reference solution (b) and the test solution, inject 20 µL of each solution three (3) times. If necessary, adjust the injection volume to between 10 µL and 20 µL in accordance with the results obtained in the test for linearity.

Report the areas of the human insulin and A21 desamido human insulin peaks in **Table 5**. Calculate the means and RSD of the human insulin peak areas. The RSD should be less than 1%. Calculate the content of human insulin plus A21 desamido human insulin and report in **Table 5**.

Attach the injection sequence and corresponding chromatograms.

Data reporting sheet (please copy for each independent assay)

System suitability tests for HPLC

Laboratory: **Date:**

HPLC equipment and detector:

Column details (column dimensions and particle size):

Report any adjustment of mobile phase, gradient or injection volumes:

Table 4: System suitability tests for HPLC

	Limits	Injection 1	Injection 2	Injection 3
Resolution between human and porcine insulin peaks	≥1.2			
Linearity of detector (reference (b) vs reference (c))	10 ± 0.5 times			

Data reporting sheet (please copy for each independent assay)

HPLC Assay – Insulin content of the candidate standard

Laboratory: **Date:**

Mass of HCl added to ampoule of candidate standard (mg): ..... mg

Mass of HCl added to bulk insulin to make Reference solution b (mg): ..... mg

Table 5: HPLC assay - insulin content of candidate standard

	Injection				
Test Solution (Candidate Standard)	**1**	**2**	**3**	**Mean**	**RSD (≤1.00%)**
Human insulin peak area					
A21 desamido peak area					
Sum of human insulin + A21 desamido peak area means = A					
	Injection				
Reference Solution (b)	**1**	**2**	**3**	**Mean**	**RSD (≤1.00%)**
Human insulin peak area					
A21 desamido peak area					
Sum of human insulin + A21 desamido peak area means = B					
Mass of bulk insulin to make reference solution (b)				**mg**	

Content of human insulin plus A21 desamido insulin per ampoule of the candidate standard is given by:

$$\frac{A \times \text{mass (mg) of HCl added to ampoule}}{B \times \text{mass (mg) HCl added to ref (b)/mass bulk insulin in ref (b)}} \times \text{insulin content of bulk material (mg /mg "as is")}$$

APPENDIX 4: PHASE 1 DATA

Table 1: Mass balance

Values are mg per 100 mg insulin API

Component	Number of labs	Estimate	Standard error	Relative standard error
Loss on drying	6	6.9095	0.1563	
Zinc	5	0.3632	0.0032	
Sodium	2	0.0244	0.0006	
Acetate	1	0.2383	0.0040	
Chloride	1	0.1600	0.0000	
Insulin plus related impurities (100 minus)		92.3047	0.1564	0.1694%
Related impurities	8	0.3130	0.0466	0.0467%
Insulin content (mg per 100 mg API)		92.0157	0.1617	0.1757%
Insulin content (mg/ampoule) Includes uncertainty due to homogeneity of fill (CV 0.1254%)		9.2034	0.0199	0.2159%
Approximate expanded uncertainty (k=2)		*9.1637*	*9.2432*	

Table 2: Nitrogen analysis

	Number of labs	Estimate	Standard error	Relative standard error
% N	3	14.4997	0.0452	
Insulin plus related impurities		92.4729	0.2880	0.3114%
Related impurities	8	0.3130	0.0466	0.0467%
Insulin content (mg per 100 mg API)		92.1834	0.2903	0.3149%
Insulin content (mg/ampoule) Includes uncertainty due to homogeneity of fill (CV 0.1254%)		9.2202	0.0313	0.3389%
Approximate expanded uncertainty (k=2)		*9.1577*	*9.2827*	

Table 3: HPLC assay - summary

HPLC Assay

	Number of labs	Estimate	Standard error	Relative standard error
Uncorrected estimate of content (without mass balance corrections)	**8**	**9.8452**	**0.0356**	**0.3618%**
Insulin content (mg/ampoule)		**9.0592**	**0.0382**	**0.4213%**
Approximate expanded uncertainty (k=2)		***8.9828***	***9.1355***	

Table 4: Individual laboratory estimate by HPLC assay

Lab	Corrected for mass balance	% discepancy vs assigned content
1	8.999	2.219
2	9.054	1.619
3	9.100	1.119
4	9.091	1.219
5	9.146	0.619
6	8.861	3.719
7	9.064	1.519
8	9.137	0.719
Mean	9.059	1.569

Appendix 2 – Phase 2 study protocol

Proposed 1st WHO International standard for insulin, human

Final study protocol – Phase 2a

Introduction

Insulin is a 51 amino acid, 5808 Da protein that is synthesised from its precursors preproinsulin and proinsulin in the beta cells of the pancreatic islets. In addition to its role as a therapeutic, measurement of insulin plays a key role in the assessment of beta cell secretion, and may improve the classification and management of diabetes in addition to monitoring for the development of insulin resistance [1-3]

The current WHO standards for insulin (human – coded 83/500, bovine – coded 83/511, porcine – coded 83/515) were established in the mid-1980's. Their potencies are defined in IU/mg. The assignment of a value to the current human insulin standard was based on a multi-method collaborative study by in vivo bioassay, hence the need for an updated standard (s) to reflect the transition of insulin internationally to a well-characterized, mass-balance assigned molecule [3, 4]. With this in mind, a new preparation of insulin has been filled into ampoules (NIBSC code 11/212) following procedures recommended by WHO [6], to produce the proposed 1st International Standard for insulin, human.

Aims of the study

It is intended that an international collaborative study is organised with expert laboratories to aid in the value assignment of the candidate International Standard, 11/212, using a mass-balance approach, and to assess its suitability to serve as a reference material for insulin immunoassay and therapeutic preparations of biosynthetic insulin. The study will be conducted in three phases:

Phase 1 – Assignment of insulin content to the candidate standard.

This phase has now been completed. The insulin content of the candidate standard was assigned based on data generated for the insulin content of the bulk active pharmaceutical ingredient (API) used to fill the candidate standard and the processing data from the definitive fill of the material into ampoules. Study participants were asked to use a mass balance approach to determine the insulin (plus A21 desamido insulin) content of the bulk API and submit this data to NIBSC for central processing.

This data was used in conjunction with data from the filling of the candidate standard (mass of bulk API filled into each ampoule and coefficient of variation for the weight of the filled ampoules) to assign the final content to the candidate standard, of 9.19 mg/ampoule (with expanded uncertainty 9.14 – 9.24 mg/ampoule, *k*=2). The uncertainty was assigned to this final value based on the combined uncertainties of the mass balance and filling data. Confirmatory data on the insulin content of the candidate standard was provided using HPLC (candidate standard vs mass-balanced bulk API).

Phase 2a – Assessment of the suitability of the candidate standard to serve as an International Standard for the calibration of diagnostic immunoassays

The performance of the candidate standard, 11/212, in current insulin immunoassays will be assessed in an international collaborative study. This will include clinical samples to allow an assessment of the likely commutability of the standard. Further details are provided below.

Phase 2b – Assessment of the suitability of the candidate standard to serve as an international standard for the calibration of secondary reference preparations used to assign potency to therapeutic preparations of insulin

Regional pharmacopoeias have established their own reference standards to be used in their respective monographs. To reduce the potential for differences in the relative assignments between different pharmacopoeias and also between batches of reference standard from the same agency, there is a requirement for a single global human insulin standard which can be used to calibrate regional/compendial standards using HPLC methods. This would improve the consistency between these standards, ideally resulting in consistent dosing to patients worldwide and more straight-forward quality control for global manufacturers.

This study protocol relates to Phase 2a of the study. The specific aims of Phase 2a of the study are:

1. To demonstrate the suitability of the preparation, 11/212, to serve as an international standard for human insulin immunoassays by examining its behaviour in current available immunoassays
2. To assess the relationships among the 1st IRR for insulin immunoassay, 66/304, local standards and the proposed 1st International Standard for insulin, human, 11/212.
3. To evaluate the likely commutability of the proposed 1st International Standard, 11/212, by examining its relationship with patient samples by immunoassay.

4. To demonstrate the stability of the candidate preparation, 11/212, by measuring activity of accelerated thermal degradation samples of the candidate standard by immunoassay.

Materials

Materials supplied to participants in collaborative study, Phase 2a

The materials to be provided to collaborative study participants are listed in Table 1. Each participant will be allocated a set of preparations based on assay capacity and sample availability. Accelerated thermal degradation (ATD) samples are not available for all participants.

The 1st IRR for human insulin immunoassay, 66/304

The 1st IRR consists of purified human pancreatic insulin, recrystallised and then dissolved in a solution of acetic acid and sucrose. The solution was dispensed into glass ampoules, lyophilised and sealed. The 1st IRR for human insulin was assigned a unitage of 3 IU per ampoule by bioassay.

The proposed 1st WHO International Standard for insulin, human, 11/212

The candidate standard 11/212 consists of highly purified, recombinant human insulin, dissolved in ddH_20 and acidified with 0.2 M HCl to dissolve. The acidified solution was neutralised to pH 7.4 with 0.2 M NaOH and the final volume made up gravimetrically to give a concentration of 10 mg/g final solution. This solution was dispensed in 1g aliquots into glass ampoules, lyophilised and sealed. The proposed 1st IS was assigned a unitage of 9.19 mg/ampoule in phase 1 of this study. Ampoules of 11/212 will be labelled with the study number and code: CS613-11/212.

Accelerated thermal degradation samples

Ampoules of the candidate IS which have been incubated at +4ºC, +20ºC, +37ºC and +45ºC for 77 months will be included in the study to assess the stability of the candidate standard by immunoassay. Not all participants will receive ATD samples. Participants receiving ATD samples will receive ampoules coded CS613 Sample A to Sample E.

Human serum and plasma samples

In order to provide information on the relationship of the candidate standard with patient samples, and its commutability with these samples by immunoassay, human serum samples will be provided for inclusion in immunoassays alongside the candidate standard 11/212, the 1st IRR 66/304 and local standards. Dependent on assay capability, 14 serum samples and 5 plasma samples (Li-Hep) can be provided and will be labelled InsSerum1 to InsSerum14 and InsPlasma1 to InsPlasma5.

Serum and plasma samples were kindly collected by Dr Gwen Wark (UKNEQAS) or purchased from First Link UK Ltd and TCS Biosciences.

These materials are only to be used for this study and in accordance with the UK Human Tissue Act or equivalent national legislation and are to be destroyed at the end of the collaborative study.

All serum and plasma samples have been tested negative for HIV-1/2, HbsAg and HCV NAT. As with all materials of biological origin, these preparations should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures.

Table 1. Material provided for Phase 2a of the Insulin collaborative study.

Preparation	**Contents**
1st IRR for human insulin immunoassay, coded 66/304	3 IU/ampoule (equivalent to 0.1 mg/amp)[1]
Candidate standard, labelled CS613-11/212	9.19 mg/ampoule (equivalent to 264.8 IU)[1]
ATD samples of 11/212, labelled CS613 Sample A to E	Assumed to be equivalent to 11/212
Human serum samples (n=14) labelled InsSerum1 to 14	0.5 ml human serum
Human plasma samples (n=5) labelled InsPlasma1 to 5	0.5 ml human plasma

[1]Conversion factor used: 1IU = 0.0347 mg (PhEur)

Handling of materials

On receipt, ampoules should be protected from light and stored at -20°C until use. Before opening, ampoules should be brought to room temperature (protected from light) to minimise moisture uptake.

It is recommended that the contents of each lyophilised ampoule are reconstituted in 1ml diluent, and then further diluted in PBS plus 0.1% BSA, or the appropriate assay buffer, to provide protein cover to prevent adsorption. A detailed protocol for reconstitution and dilution of the standards is provided in Appendix 1.

It is important that upon delivery, serum and plasma samples are stored at -20ºC or below until they are required. Serum samples should be thawed at room

temperature and mixed well prior to assay. Plasma samples should be thawed by placing in a water bath at 37ºC for 5-10 mins to minimise the formation of cryoprecipitates. Centrifuge at 250 *xg* for 10 mins and remove supernatant for testing. Please do not re-freeze samples. Use a fresh aliquot for each run.

Tests requested

Participants are requested to carry out the assay method(s) normally used in their laboratory and to perform **three independent runs.** An independent run consists of the measurement of one set of test samples ('dilutions') prepared from previously unopened ampoules and one set of serum samples (n=14) and plasma samples (n=5) which have been thawed specifically for that run. An independent run will use a single calibrated kit, integral or plate as required for your method.

Participants are requested to prepare dilutions of the ampouled preparations and to measure, in triplicate, the insulin concentration of these and the serum/plasma samples in each run.

Participants also receiving accelerated thermal degradation samples CS613 Sample A to E can perform each run of these samples independently of CS613-11/212, 66/304 and serum/plasma samples if desired.

Each independent run will also include the measurement of the kit calibrators or in-house standards for that method.

Common test sample concentrations

It is important that the same dilutions of each ampouled preparation are measured by all participants in order to evaluate the activity of the candidate standard by different immunoassay methods. Therefore, participants are requested to prepare and measure the insulin concentration of 7 core dilutions of 66/304 and 11/212 which are common to all participants. These dilutions are 288, 144, 72, 36, 18, 9 and 4.5 µIU/ml, and further detail on the preparation of these is provided in Appendix 1.

However, please note that additional samples should be prepared as required to ensure that a minimum of **five points in the linear part of the dose response curve are measured** in addition to the common dilutions as listed above.

Data submission

Participants are requested to provide details of the assay method used, including dilution steps, together with all raw data e.g. counts for each sample, in electronic format if possible. Participants' own calculated estimates of insulin concentration are also requested, along with details of kit specific conversion factors if applicable. A sample table for data reporting is provided in Appendix 1.

Report

A preliminary report will be prepared and circulated to all participants for comment before submission to the Expert Committee on Biological Standardization of WHO. In the report, participating laboratories will be identified by a laboratory number only and any requests to treat information in confidence will be respected.

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Appendix 1

Assay buffer

For dilution steps below, please use PBS plus 0.1% bovine serum albumin (BSA) or your appropriate assay buffer, ensuring protein cover is provided to prevent adsorption.

Standard and sample processing

The following provides details on the reconstitution of the standards, along with example dilution steps to generate the working stock solution of the standards for use in the assay. These example dilution steps, or your own in-house dilution methods may be used, but please provide all details of reconstitution and dilution steps taken in your report.

A fresh ampoule of 11/212 should be used for each test. Due to limited stocks of 66/304, only one ampoule can be provided to participants that must be aliquoted and used in all tests.

A. Reconstitution and dilution of candidate standard 11/212

1. Before opening, ampoules should be brought to room temperature to minimize moisture uptake.
2. Reconstitute each ampoule in 1ml ddH_2O, then dilute further in assay buffer to give a 1 mg/ml stock solution. This is equivalent to a 28.8 IU/ml stock solution using the conversion factor 1IU = 0.0347 mg
3. Dilute the solution in step 2 to provide a 20 ng/ml (equivalent 576 µIU/ml) working stock solution. You may use the method normally in use in your laboratory. For example, the 1mg/ml stock solution may be diluted 1:1000 (e.g. 10 µl to 10 ml assay buffer) to provide a 1 µg/ml stock solution, and then further diluted 1:50 (e.g. 100µl to 4900µl assay buffer) to provide the 20ng/ml working stock solution.
4. The working stock solution at 20ng/ml (or 576 µIU/ml) will form dilution 1 and the solution from which serial dilutions should be made.
    - Prepare serial dilutions of this working stock solution to provide dilutions 2 to 10. Table A1 below provides the full details of the dilutions and their expected concentrations.
    - To enable comparison across different immunoassays at the same dilution point, participants are asked to include the **7 core concentrations highlighted in bold** which should be included in **all assays**. If assay space permits, additional concentrations should be included.

B. Reconstitution and dilution of 66/304

1. Before opening, the ampoule should be brought to room temperature to minimize moisture uptake.
2. Reconstitute the ampoule in 1 ml ddH_2O to give 3 IU/ml.
3. Add solution in step 2 to 4.21 ml assay buffer to provide a 576 mIU/ml stock solution. Due to limited stocks of 66/304, 1 ml aliquots of this 576 mIU/ml stock solution should be prepared and frozen at -20ºC for use in subsequent tests. Please freeze all aliquots of 66/304 together, and thaw one when required to ensure the standard is used at the same freeze-thaw cycle in each run.
4. Dilute the solution from step 3 to provide a 576 µIU/ml working stock solution. You may use the method normally in use in your laboratory. For example, the 576 mIU/ml stock solution may be diluted 1:1000 (e.g. 10µl to 10ml). The 576 µIU/ml working stock solution will form dilution 1 and the solution from which serial dilutions should be made.
    - Prepare serial dilutions of this working stock solution to provide dilutions 2 to 10. Table A1 below provides the full details of the dilutions and their expected concentrations.
    - To enable comparison across different immunoassays at the same dilution point, participants are asked to include the **7 core concentrations highlighted in bold** which should be included in **all assays**. If assay space permits, additional concentrations should be included.

Table A1 Standard dilutions table

11/212 dilution	**Concentration (ng/ml)**	**66/304 dilution**	**Concentration (µIU/ml)**
Step 3	1000	Step 3	576000
Step 4/Dilution 1	20	Step 4/Dilution 1	576
Dilution 2	**10**	**Dilution 2**	**288**
Dilution 3	**5**	**Dilution 3**	**144**
Dilution 4	**2.5**	**Dilution 4**	**72**
Dilution 5	**1.25**	**Dilution 5**	**36**
Dilution 6	**0.625**	**Dilution 6**	**18**
Dilution 7	**0.3125**	**Dilution 7**	**9**
Dilution 8	**0.156**	**Dilution 8**	**4.5**
Dilution 9	0.078	Dilution 9	2.25
Dilution 10	0.039	Dilution 10	1.125

C. Preparation of serum samples InSerum1 to InsSerum14 and plasma samples InsPlasma1 to InsPlasma5

Upon arrival, please store all serum and plasma samples at -20ºC or below until use. Serum samples should be thawed at room temperature and mixed well prior to assay. Plasma samples should be thawed by placing in a water bath at 37ºC for 5-10 mins, then centrifuged at 250 *xg* for 10 mins. The supernatant should then be removed for testing. Please do not re-freeze samples. Use a fresh aliquot for each run.

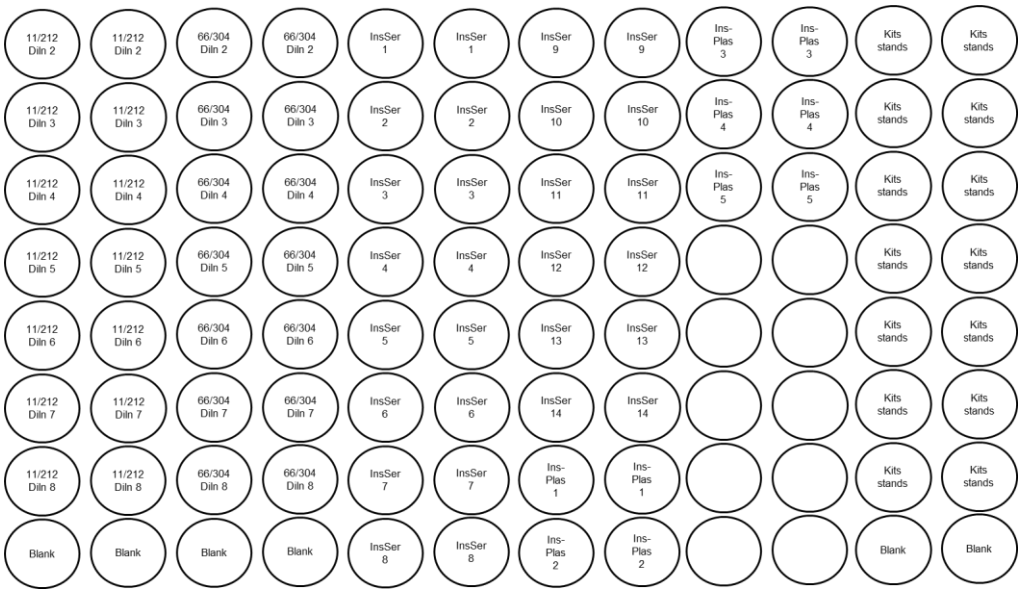
D. Assay design and plate layout

Alongside local standards and controls, each assay should include 66/304 and the candidate standard 11/212, each at a minimum of five dose levels in the linear part of the dose-response curve as described in section A and B above. Each assay should also include the patient serum/plasma samples. All samples should be tested in triplicate according to the in-house method. To enable all samples to be incorporated into a standard 96 well ELISA plate format, standard samples (11/212, 66/304) and plasma/serum samples can be run in duplicate alongside kit controls and local standards.

To enable us to gather data regarding inter and intra-assay variability within each laboratory, participants are requested, where possible, to perform at least three independent assays with the samples provided. Due to limited stocks of 1st IRR 66/304, only 1 ampoule can be provided. In this case, perform reconstitution as described in B) above, making aliquots of the 576 mIU/ml stock solution and freezing at -20ºC for use in Assay 2 and 3.

Standards 11/212 and 66/304 should be included on all plates alongside local standards.

Suggested plate map for ELISA plate format



E. Data reporting

Estimates of the insulin content of the candidate standard 11/212, the 1st IRR 66/304 and the patient samples should be calculated in comparison with the in-house assay kit standard.

Participants are requested to provide details of the assay method used, including dilution steps, together with all the raw data e.g. counts for each sample in electronic format (excel file) if possible. Participants' own calculated estimates of insulin concentration are also requested. A sample table for data reporting is provided below in Table A2.

Table A2 Sample data reporting table

Assay Run No.	***Platform:***				***Method:***			
	RLU/Absorbance Units/Counts				***Reported Insulin concentration (µIU/ml; ng/ml)***			
****Sample***	***1***	***2***	***3***	***Avg***	***1***	***2***	***3***	***Avg***
Baselines								
Kit standard 1								
Kit standard 2								
Kit standard 3								
Kit standard 4								
Kit standard 5								
66/304 dil 2								
66/304 dil 3								
66/304 dil 4								
66/304 dil 5								
66/304 dil 6								
66/304 dil 7								
66/304 dil 8								
11/212 dil 2								
11/212 dil 3								
11/212 dil 4								
11/212 dil 5								
11/212 dil 6								
11/212 dil 7								
11/212 dil 8								

InsSerum1								
InsSerum2								
InsSerum3								
InsSerum 4								
InsSerum 5								
InsSerum 6								
InsSerum 7								
InsSerum 8								
InsSerum 9								
InsSerum 10								
InsSerum 11								
InsSerum 12								
InsSerum 13								
InsSerum 14								
InsPlasma 1								
InsPlasma 2								
InsPlasma 3								
InsPlasma 4								
InsPlasma 5								

* Expand table for ATD coded ampoules and/or additional dilutions as required