EXPRESS COMMITTEE ON BIOLOGICAL STANDARDIZATION
Geneva, 17 to 20 October 2017

Proposed WHO Reference Reagent for Activated Blood Coagulation Factor X (FXa), Human

Craig Thelwell¹, John Hogwood¹, Sarah Daniels¹, Peter Rigsby²

¹Biotherapeutics Group, Haemostasis Section and ²Biostatistics Section, National Institute for Biological Standards and Control, South Mimms, Herts EN6 3QG, UK

NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by 18 September 2017 and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: Dr C M Nübling at email: nueblingc@who.int

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Summary

Approval was obtained from the Expert Committee on Biological Standardization of WHO to prepare a WHO Reference Reagent (RR) for Activated Blood Coagulation Factor X (FXa), Human. The RR is proposed as a replacement for the current Non-WHO Reference Material for FXa (75/595), of bovine origin. A candidate material was donated by a manufacturer and was formulated, lyophilized and coded 15/102. The proposed WHO RR was calibrated against 75/595 using a direct chromogenic assay with a potency estimate for 15/102 based on results from 12 independent assays from two laboratories. Intra-laboratory variability was low (GCV<4%) and laboratory GM estimates showed good agreement (6.5 and 6.8 units per ampoule). The overall geometric mean was used to potency assign the candidate RR (15/102), which was determined to be 6.7 units per ampoule.

Introduction

Blood coagulation factor Xa (FXa) is a trypsin-like serine protease. FXa plays a critical role in the coagulation cascade, holding a central position that links the extrinsic and intrinsic pathways. FXa catalyses the proteolytic conversion of prothrombin to thrombin, in complex with Factor Va and phospholipids, in the presence of calcium. FXa is an essential component of FEIBA (factor eight inhibitor bypassing activity), an activated prothrombin complex concentrate (APCC) used in the treatment of haemophilia patients with inhibitory antibodies against FVIII [1]. FEIBA primarily consists of the zymogen forms of FII, FVII, FIX and FX, along with trace amounts of their activated forms. The presence of FXa is required to form a FII-FXa complex, which triggers an immediate thrombin generation, prompting initial fibrin clot formation and thrombin-mediated feedback reactions leading to the activation of other coagulation components [2].

In the absence of an international reference standard for FXa, direct measurements of FXa activity in FEIBA have been made relative to working standards originally calibrated against the non-WHO Reference Material for Blood Coagulation Factor Xa (75/595), sourced from bovine plasma. This bovine FXa reference material is available from the National Institute for Biological Standards and Control (NIBSC) though it was not formulated and filled by NIBSC. The bovine FXa reference material (75/595) was prepared and freeze-dried into sealed ampoules in 1975 by an external group, organised by Jackson et al., and arbitrarily assigned a potency of 1.0 Unit per ampoule [3]. With no information on the uniformity of the fill of 75/595, or on its stability, the suitability of the continued use of this reference material is in question and a replacement for 75/595 is proposed.

Although measurement of FXa in FEIBA is the primary regulatory use for a FXa reference standard, 75/595 is used routinely by several other laboratories to calibrate local standards. Potential future uses for a FXa reference material include standardising the measurement of FXa as a contaminant in non-activated products such as prothrombin complex concentrates (PCC), used in the reversal of anticoagulant therapy, and a recently licensed Factor X concentrate product used in the treatment of congenital FX deficiency. Standardising the biological activity of direct FXa inhibitors, used in anticoagulation therapy, is another significant potential use which would require a human FXa standard due to potential differences in the active site
inhibition between bovine and human FXa. The use of a bovine standard for the measurement of human FXa is also restrictive in terms of assay discrepancy, there being significant differences between the chromogenic and clotting activities of bovine and human FXa. It is therefore proposed that the bovine FXa standard (75/595) is replaced with a preparation derived from human plasma.

The development of an International Standard for human FXa would require a large scale study with representative assay methods for its intended use. Since the full range of intended uses for this reference material are not yet known, it is proposed to establish a Reference Reagent (RR) as an interim measure to evaluate its potential. In the absence of a commonly recognised unit definition for FXa activity, and in the interests of continuity with the existing bovine FXa unit, it is proposed to calibrate the human FXa RR relative to 75/595 using a direct chromogenic activity assay. This choice of assay system ensures consistency with current FXa measurements in FEIBA and avoids any discrepancies between the interactions of human and bovine FXa with the other components of the coagulation system.

The calibration of a candidate WHO RR for Activated Factor X (FXa), Human is outlined in the current report.

Materials

One manufacturer kindly provided the bulk material for the preparation of the candidate WHO RR for FXa, Human. The starting material, a concentrate of human plasma-derived factor X, was activated using Russell’s viper venom-factor X activator (RVV-X). Factor Xa was purified from RVV-X by affinity chromatography using Benzamidine-Sepharose. The eluate was desalted and reformulated into 20 mM Hepes buffer with 1% human albumin, pH 7.4. Two purification runs were performed and provided as frozen aliquots, total volume 555 ml containing approx. 89000 units FXa. The formulation, dilution and freeze-drying conditions for the definitive fill were based on the results of small scale trial fills, and on experience with previous formulations. For the definitive fill the material was thawed and diluted to a final concentration of approx. 7 U/ml (based on the manufacturer’s potency estimates and on NIBSC analysis) relative to the bovine factor Xa NIBSC reference preparation (75/595). The bulk material was diluted in 20 mM Hepes buffer (pH 7.4) containing 10 mg/ml human albumin, 5 mg/ml trehalose and 150 mM NaCl. 5 ml DIN ampoules were filled with 1 ml aliquots of the diluted material, lyophilised following WHO procedures, and coded 15/102. Filling and lyophilisation was carried out at NIBSC (Potters Bar, UK) where the ampoules are also stored at -20 °C. Further details of the ampouled material for 15/102 are given in the table below.

| Activated factor X (FXa), human 15/102 |
|-------------------------------|---------------------|
| Number of containers | 11739 |
| Mean fill mass g (CV) | 1.0093 (0.13%) |
| Mean dry weight g (CV) | 0.02987 (3.37%) |
| Mean residual moisture % (CV) | 0.205 (52.28%) |
| Mean oxygen head space % (CV) | 0.12 (43.85%) |
Participants

Two independent laboratories participated in the study: Sarah Daniels, Biotherapeutics Group, NIBSC, Potters Bar, UK; Peter Gartner, Baxalta, Vienna, Austria.

Results and discussion

Assay methods and study design
The aim of the study was to assign a potency value to the candidate WHO RR for Activated Factor X (FXa), Human (15/102), relative to the Non-WHO Reference Material for Coagulation Factor Xa (75/595).

Study participants were provided with six ampoules each of 75/595 and 15/102. Each laboratory performed their own in-house assay method to measure the chromogenic activity of each preparation in six independent assays, using fresh ampoules in each. A minimum of three dilutions of each preparation and the inclusion of two or more replicates of each dilution within each assay was requested. Approximate potencies of each of the samples were provided to allow an appropriate dilution regime. Participants were requested to return their results as kinetic rates or endpoint readings.

Statistical analysis
Potency estimates for the candidate WHO RR (15/102) were calculated relative to the Non-WHO Reference Material (75/595) using a slope ratio model with no transformation of the assay response. All calculations were performed using the software program Combistats [4]. For each laboratory, results from all assays were combined to generate an unweighted geometric mean (GM) potency. Variability between assays has been expressed using geometric coefficients of variation (GCV = \( \{10^{s-1}\} \times 100\% \) where s is the standard deviation of the \( \log_{10} \) transformed potencies).

Summary of results
Potency estimates were calculated based on 12 independent assays from the 2 laboratories that received samples. A single assay from each laboratory showed significant (p<0.01) deviations from the fitted model (linearity and common intercept), but this appeared to result from underestimation of residual error and the acceptability of the model was confirmed following visual inspection of the plotted data.

The overall geometric mean potency for the candidate WHO RR for Factor Xa (15/102) was 6.7 units per ampoule, which was consistent with the target potency based on the activity of the bulk material. Intra-laboratory GCVs for both laboratories were below 4%. The results are presented in Table 1.
Table 1. Potency estimates (U/ampoule) for 15/102.

<table>
<thead>
<tr>
<th>Lab</th>
<th>Estimates</th>
<th>GM</th>
<th>GCV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.7</td>
<td>6.5</td>
<td>3.7%</td>
</tr>
<tr>
<td>2</td>
<td>6.7</td>
<td>6.8</td>
<td>3.0%</td>
</tr>
<tr>
<td>Overall GM</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The low variation within the two participating laboratories and the good agreement between them provides confidence in the continuity of the unit of FXa activity from 75/595 to the candidate RR (15/102), which should ensure the continuity of FXa measurement in FEIBA and of other working standards calibrated relative to 75/595. Preparation 15/102 is therefore proposed for establishment as the WHO RR for Factor Xa.

Proposal

Preparation 15/102 is proposed as the WHO Reference Reagent for Activated Factor X (FXa), Human, with a potency of 6.7 units per ampoule.

Future development of an International Standard

Establishing a WHO RR for FXa will provide an opportunity to investigate the development of a WHO International Standard (IS). The unit of activity defined by the RR provides continuity with the unit established by the bovine FXa reference standard (75/595) and the potential basis for establishing a future international unit (IU). To achieve this, the utility of the RR for potency estimation of human FXa must be assessed in the full range of assay methods in contemporary use. It is therefore proposed to follow up on orders placed for the RR with a questionnaire to discover the intended purpose for the standard, and the assay method(s) to be used. Feedback may then be requested from end users on the usefulness of the RR for this purpose and on any issues arising with any particular assay system. This information may then inform the nature of a future collaborative study to establish a WHO IS for FXa, Human.

Participants’ response

All participants (2/2) agreed with the conclusion in the report that preparation 15/102 be proposed as the WHO Reference Reagent for Activated Factor X (FXa), Human, with a potency of 6.7 units per ampoule. No specific comments were received.

Expert review by the Control of Anticoagulation Subcommittee of the SSC/ISTH

Expert opinion was sought from a panel of experienced scientists selected by the Chair of the Control of Anticoagulation Subcommittee of the SSC of the ISTH. Responses were received from 8 experts and all agreed with the proposals. The proposal to endorse preparation 15/102 as
the WHO Reference Reagent for Activated Factor X (FXa), Human, was approved at the SSC Board Meeting held in Berlin, Germany, on 9 July 2017.

One reviewer comment was received on the study:

“I thought the proposal was well-justified and the study was well-executed. I approve. My only question is one of ignorance: are two laboratories sufficient (i.e. is this the industry standard or should validation occur in a larger number of laboratories?)”

To address this question, which is very pertinent, there are a number of considerations. In terms of acceptability to WHO it is valid to only use only two laboratories to calibrate a Reference Reagent, and this is addressed in Annex 2 of the WHO Technical Report Series, No 932 (Recommendations for the preparation, characterization and establishment of international and other biological reference standards):

“…it is sufficient for a limited number of laboratories to examine a characterized product and agree to the assignment of potency as expressed in units. As a minimum, the bulk material used in the preparation should have been shown to retain biological activity consistent with the assigned unitage by a competent laboratory, for example the manufacturer, and this biological activity should have been confirmed by an independent laboratory, preferably a WHO collaborating laboratory.”

In terms of justification, the decision to only use two laboratories was based on the primary requirement for establishing a new reference material for FXa. The existing standard, 75/595 (bovine FXa), has no official status and there is no information on its uniformity or stability. 75/595 does however define the unit of activity for FXa in FEIBA, and FXa measurement in FEIBA is a regulatory requirement. Continuity of the unit defined by 75/595 was therefore the main priority in establishing a new reference material and this was best achieved using a simple chromogenic assay to commute the unit from bovine FXa to human FXa. Agreement between the FEIBA manufacturer and an independent laboratory (NIBSC) was I believe the best way to achieve this. The chromogenic assay used in the study is not routinely performed by other laboratories, and at this stage it would not have been possible to include a wide range of assay methods, e.g. clotting methods, due to the discrepancy between bovine and human FXa. Having an established WHO Reference Reagent will make it possible to investigate applicability with a wide range of assay methods, and involve a larger number of laboratories, and we will offer to work with manufacturers and other interested laboratories to help with this process.

**Stability of the proposed candidate material 15/102**

**Long term stability**

Predictions for the long term stability of the proposed candidate material (15/102) will be assessed over time by monitoring the Factor Xa potency of ampoules stored under accelerated degradation conditions. Ampoules of the proposed candidate preparation were stored at a range of temperatures immediately following lyophilisation (-150 ºC, -70 ºC, -20 ºC, +4 ºC, +20 ºC, +37 ºC, +45 ºC and +56 ºC).
To provide an indication of long term stability, two ampoules of 15/102 from each storage temperature were assayed by NIBSC following storage under accelerated degradation conditions for eight months. Potency estimates were obtained for the degradation samples (+20 °C, +37 °C, +45 °C and +56 °C) relative to ampoules stored at -20 °C. In addition to the chromogenic method used in the calibration exercise, two different clotting methods were also used: prothrombin time (PT) and activated partial thromboplastin time (APTT). The results are presented in the table below, expressed as a percentage of the potency result for the -20 °C samples. Each result is based on a combined potency from two ampoules assayed separately in duplicate.

<table>
<thead>
<tr>
<th>Assay method</th>
<th>% activity relative to -20 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+20 °C</td>
</tr>
<tr>
<td>Chromogenic</td>
<td>107.2 %</td>
</tr>
<tr>
<td>PT</td>
<td>98.6 %</td>
</tr>
<tr>
<td>APTT</td>
<td>97.6 %</td>
</tr>
</tbody>
</table>

The results suggest that candidate material 15/102 is very stable. There was no observed loss of potency using the chromogenic assay for any of the storage temperatures. The results of the PT and APTT clotting assays were successfully fitted to the Arrhenius Equation and the prediction for the % loss of activity per year for samples stored at -20 °C (normal storage conditions) was 0.033 % (upper 95 % confidence limit 0.103 %) for the PT assay and 0.043 % (upper 95 % confidence limit 0.183 %) for the APTT assay. Stability monitoring is ongoing and will be assessed for longer time periods at lower storage temperatures.

**Bench stability following reconstitution**

To provide an indication of the stability of the proposed candidate material throughout a typical assay period, the potency of the proposed candidate (15/102) was monitored following reconstitution with distilled water, as recommended in the Instructions for Use. On the day of the study two ampoules were reconstituted and the contents transferred into two stoppered plastic tubes, labelled T1 and T2. Each sample was stored on melting ice and assayed for FXa activity relative to a freshly reconstituted ampoule at time 0, 2 hours, 4 hours and 6 hours. All assays were performed at NIBSC using a PT clotting method. FXa potency estimates are provided in the table below, based on the combined result of two assays and expressed as a percentage of the potency estimate calculated at time zero.

| % activity relative to fresh ampoule at time zero (95 % confidence interval) |
|-------------------------|-----------------|-----------------|
|                         | 2 hours         | 4 hours         | 6 hours        |
|                         | 90.93 % (79.5 – 104.1) | 94.0 % (76.2 – 116.1) | 87.6 % (80.4 – 95.5) |
This limited data suggests the material is stable over a normal assay period, and supports a recommendation that potency assays should be completed within four hours of reconstitution of the standard. The IFU (instructions for use) for 15/102 will reflect this; however it will be recommended that end users investigate stability following reconstitution for their own storage and assay conditions.

References

4  CombiStats Version 5.0. EDQM - Council of Europe; 2013.

Acknowledgments

We are extremely grateful to Shire (formerly Baxalta) for the donation of human FXa used to prepare the candidate reference material, and to the following individuals and organisations for their invaluable contribution to the study:

The members of the project team from the Centre for Biological Reference Materials at NIBSC, for development work on filling and organisation of sample shipping.

The Control of Anticoagulation SSC Subcommittee of the ISTH.

Participants who took part in the study and to everyone else involved who is not identified personally.
Appendix 1. Instructions for use

WHO Reference Reagent
Activated Blood Coagulation Factor X (F-Xa), Human
NIBSC Center 15/922
Instructions for use
(Versions 1.05, Date__)

1. INTENDED USE

The WHO Reference Reagent for Activated Blood Coagulation Factor X (F-Xa) (15/922) was established by the Expert Committee on Biological Standardization of the World Health Organization in October 2017, and a report of the collaborative study is available from WHO, reference number WHO/Bi/2017/30X.

The intended use of this preparation is to standardize F-Xa potency measurements in therapeutic APTT (activated partial thromboplastin complex) reagents. The reference reagent may also be used to standardize F-Xa measurements for other therapeutic products or to standardize the biological activity of F-Xa inhibitors. The outcome of any investigation may inform the nature of a future collaborative study to establish 15/922 as a WHO International Standard for F-Xa.

A potency of 6.7 unitampoule was assigned in a collaborative study based on chromogenic assays relative to the Non-WHO Reference Material for Blood Coagulation Factor Xa (29550), a lyophilized preparation of F-Xa.

2. CAUTION

This preparation is not for administration to humans or animals in the human food chain.

The preparation contains material of human origin, and either the final product or the source materials, from which it is derived, have been tested and found negative for HBsAg and HCV RNA. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and disposed of according to your own laboratory’s safety procedures. Such safety precautions should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The assigned potency of this preparation is 6.7 unitampoule.

4. CONTENTS

The bulk material used to prepare this reference reagent for F-Xa was donated by one manufacturer as a frozen solution of human plasma-deficient factor X, activated using Stuart’s reagent containing Xa activator (RVV-X) and purified by affinity chromatography using Bacteriophage-Septarins. The material was thawed and diluted in a final concentration of 2 units/ml based on F-Xa potency estimated in 20 mM Hepes buffer (pH 7.5), 5 mM magnesium chloride, and 150 mM NaCl. A total of 1173.5 mg DIN ampoules were filled at 1 ml aliquots of the diluted material with a mean filling weight of 1.005 g (CV = 0.13%). Freeze-drying was followed by WHO reprocessing to product ampoules with a mean dry weight of 0.295 g (CV = 0.37%) and a residual moisture of 6.20% (CV = 0.40%).

5. STORAGE

Upon receipt unpackaged ampoules should be stored in the dark at or below -29°C.

Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an “easy-open” coloured stress point, where the narrow ampoule meets the wider ampoule body. To open the ampoule gently to collect the material at the bottom (labelled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker against the ampoule stem between the thumb and first finger of the other hand, apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

Care should be taken to avoid cuts and project glass fragments that might enter the eyes, for example by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution. Allow the ampoule to reach ambient temperature before opening and reconstitute with 1.0 ml distilled or deionized water.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials. It is the policy of WHO not to assign an expiry date to their international reference materials and they remain valid with their assigned potency until withdrawn or amended.

Predictions on long-term stability are made by measuring ampoules stored under accelerated storage conditions over time.

Based on the results of a stability test it is advised that ampoules are stored on wet ice for reconstitution, and assayed should be completed as soon as possible and within 4 hours.

9. REFERENCES

A report of the collaborative study to calibrate the standard is available from WHO, reference number WHO/Bi/2017/30X.

10. ACKNOWLEDGEMENTS

We are grateful to Shire (Australia) for the donation of the starting material, to the collaborative study participants, and to the Control of AntiCoagulant Subcommittee of the Standardization and Scientific Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH).

11. FURTHER INFORMATION

Further information can be obtained as follows:
NIBSC: material.capture@nicb.org
WHO: biologics@who.int

WHO Biologics Standards:
https://www.who.int/biologics/standards

JCTLM: Higher order reference materials:
http://www.mbn.org/main/reference\jtclm

Derivation of international Units:

Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx

NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx
12. CUSTOMER FEEDBACK
Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to endusers@nibsc.org

13. CITATION
In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET
Classification in accordance with Directive 2000/54/EC, Regulation EC No 1272/2008: Not applicable or not classified

<table>
<thead>
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<th>Physical and Chemical properties</th>
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<tr>
<td><strong>Property</strong></td>
<td><strong>Value</strong></td>
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<tr>
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</tr>
<tr>
<td>Hygroscopic:</td>
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<tr>
<td>Irritant:</td>
<td>No</td>
</tr>
<tr>
<td>Flammable:</td>
<td>No</td>
</tr>
<tr>
<td>Other (specific):</td>
<td>Contains material of human origin</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Toxicological properties</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Effects of inhalation:</td>
<td>Not established, avoid inhalation</td>
</tr>
<tr>
<td>Effects of ingestion:</td>
<td>Not established, avoid ingestion</td>
</tr>
<tr>
<td>Effects of skin absorption</td>
<td>Not established, avoid contact with skin</td>
</tr>
</tbody>
</table>

**Suggested First Aid**

- **Inhalation**: Seek medical advice
- **Ingestion**: Seek medical advice
- **Contact with eyes**: Wash with copious amounts of water. Seek medical advice
- **Contact with skin**: Wash thoroughly with water

**Action on Spillage and Method of Disposal**

- Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant, rinse area with an appropriate disinfectant followed by water
- Absorbent materials used to treat spillage should be treated as biological waste

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistency between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/about_UKTerms_and_Conditions.aspx or upon request by the Recipient) (“Conditions”) apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient’s attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMERS USE ONLY

- **Country of origin for customers purposes**: United Kingdom
- **Defined as the country where the goods have been produced and/or sufficiently processed to be classified as originating from the country of supply for example a change of state such as freeze-drying**
- **Net weight**: 29 mg
- **Toxicity Statement**: Not toxic
- **Veterinary certificate or other statement if applicable**: Attached: No