EXPERT COMMITTEE ON BIOLOGICAL STANDARDIZATION
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Collaborative study for the establishment of the Second International Standard for Gramicidin

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WHO/BS/08.2100
Page 2

Summary
An international collaborative study was organised to establish the second World Health Organization (WHO) International Standard (IS) for gramicidin. The report presents this study in which six laboratories from different countries participated. Potencies of the candidate material were estimated by microbiological assays with sensitive micro-organisms. To ensure continuity between consecutive batches, the first IS for gramicidin was used as standard.

This report provides details about the material donated by a manufacturer, the processing involved to establish a candidate batch and the analytical controls to assess its quality. It describes the statistical analysis of the results, the conclusions made thereof and the recommendation to the WHO Expert Committee on Biological Standardization (ECBS).

It is proposed that the *Second WHO International Standard for Gramicidin* (EDQM internal code ISA_28168) be assigned an antimicrobiological activity of \textbf{1070 IU per milligram of substance}.

Introduction
Gramicidin is a heterogeneous mixture of linear polypeptides isolated from the fermentation broth of *Brevibacillus brevis* Dubos. The polypeptides exhibit ß-helix structures which dimerize to span lipid bilayer thus creating ion channels. The resulting increased bacterial cell wall permeability to small inorganic ions is the basis of the antimicrobial activity.

The first IS for gramicidin was established by the WHO in 1964 on the basis of an international collaborative study [1]. It was assigned with a potency of 1000 International Units per mg (IU/mg), each ampoule containing approximately 55 mg.

As stocks of the first IS were becoming exhausted, the European Directorate for the Quality of Medicines & HealthCare (EDQM), was requested by the ECBS to undertake appropriate steps for its replacement by the establishment of a new batch.

Bulk material, processing and stability
Candidate bulk material was kindly donated by Alpharma ApS, Copenhagen (Denmark). Several separate bottles each containing 0.5 kg of gramicidin of the same batch of current pharmaceutical grade appropriate for therapeutic use were received by the EDQM in April 2006. Upon receipt, the bulk material was stored at +4°C before processing. The candidate material was claimed by the manufacturer to comply with the quality standards of the relevant monographs of both the European Pharmacopoeia and the United States Pharmacopeia. A certificate of analysis was provided in the batch documentation.

Filling
All powder weighing and filling was carried out in glove boxes under a controlled atmosphere by use of argon gas.

The powder of one bottle was allowed to equilibrate at room temperature and subsequently submitted to homogenisation in a Turbula mixer. Twelve weighings of 30 g were distributed in separate containers which were subsequently sealed, protected from light and stored at -20°C. The filling campaign was organised over five consecutive days from 5 to 9 November 2007. Prior to any further processing of the gramicidin powder, containers were stored overnight unopened under the glove box to enable room temperature equilibration.
Production of a suitable “reference standard” for monitoring purposes

WHO IS are primary reference materials and as such cannot be tested against higher order reference standards. As a consequence, real time stability studies are not usual practice and in many cases, stability of WHO IS were assessed by means of accelerated degradation studies.

In the documentation supplied with the batch, stability was confirmed for a period of 36 months. Over this period, the manufacturer reported no decrease in microbiological activities.

Nevertheless, it was decided to store some of the powder at −80°C and to use it, at regular intervals in the future, to assess the potency of vials stored at −20°C, the customary storage temperature of the WHO IS batch for gramicidin. Consequently, it was decided to start the filling process by weighing 100 mg in white glass ampoules from the first 30 g container. A total of 220 ampoules were prepared. They were immediately sealed by fusion. The entire batch was stored at −80°C and is identified under the EDQM internal number 32665.

Production of the second WHO IS for gramicidin candidate batch

The filling was carried out in the EDQM facilities. Fillings were organised in morning and afternoon sessions and traced by sub-batch numbering. A total of 2020 vials were filled with 100 mg of gramicidin, closed with an inert rubber stopper and sealed. All vials were stored at -20°C.

Quality control on bulk and final batch

Conformity of the bulk

Prior to starting the filling process, 20 g were sampled from the bulk. This material was submitted to physico-chemical analysis according to the European Pharmacopoeia monograph, to confirm compliance. The results obtained using the analytical methods described under “Ultraviolet and Visible Absorption Spectrophotometry, Thin Layer Chromatography, Composition, Related Substances, Loss on Drying and Sulfated Ash” were in good agreement with those of the certificate of analysis provided by the manufacturer. The bulk was therefore considered suitable for further processing.

Homogeneity of powder fillings

Filling was carried out to a nominal content of 100 mg with a balance connected to a recording device. Mean filling weights and relative standard deviations (RSD) were calculated for each session. No significant variability was observed between sessions and the overall mean filling weight was 100.78 mg (RSD 0.11 %). These results supported the assumption that there was no heterogeneity in weight between vials as a result of filling sessions fractionated into half days.

Sampling design

During each half day filling session, 3 filled vials were sampled at the beginning, middle and end of the session. Each vial was individually tagged for unambiguous identification and stored at −20°C until subsequent analysis.

Quality control

From the above pool of samples, the 3 vials collected at the middle of each filling session were tested for their water content by the loss on drying (LOD) method [as described in the European Pharmacopoeia (Ph. Eur.) general chapter 2.2.32 loss on drying].
Results

Three assays were carried out (1 to 3). Samples from different half day filling sessions (Monday morning to Friday afternoon) are numbered 1 to 10. Each assay included 1 sample from each session. To eliminate as much as possible the effect of the order of weighing, the samples were tested using a rotating design. The order of weighing was 3, 10, 7, 4, 1, 8, 5, 2, 9, 6 in the 1st assay and the same order (but rotated) was used for assays 2 and 3, starting with session 5 and 4 respectively. A summary of results is given in Table 1 and a plot of the results is presented in Figure 1.

Table 1 – Overview of results loss on drying (in%)

<table>
<thead>
<tr>
<th>Session</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
<th>Mean</th>
<th>SD</th>
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<td>0.414</td>
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<td>2</td>
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<td>0.755</td>
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<td>0.605</td>
<td>0.825</td>
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<td>7</td>
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<td>9</td>
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<td>10</td>
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<td>0.828</td>
<td>0.720</td>
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<tr>
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<td>0.111</td>
<td>0.087</td>
<td>0.083</td>
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</table>
An analysis of variance was carried out in which the difference between assays (1 to 3) and between sessions (1 to 10) were modeled as classes and the order of weighing was modeled as a linear effect. The results are given in Table 2.

<table>
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<tr>
<th>Source</th>
<th>DF</th>
<th>SS</th>
<th>Mean Square</th>
<th>F</th>
<th>Pr&gt;F</th>
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<tbody>
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<td>0.01269200</td>
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<td>0.1796</td>
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<td>0.02023779</td>
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<td>0.0207</td>
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<td>0.10127763</td>
<td>15.63</td>
<td>0.0001</td>
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</table>

The order in which the samples were tested does not have a significant effect. The largest variation is explained by systematic differences between assays. The largest difference is observed between assay 2 and assay 3 where the mean LOD was 0.629% and 0.828% respectively. There also appears to be a systematic difference between samples from different sessions, but this effect is less important than the difference between assays. The largest difference is found between samples from session 1 (Monday morning) and session 4 (Tuesday afternoon) with mean losses of 0.583% and 0.849% respectively. This difference is practically irrelevant so it can be considered that the batch is sufficiently homogeneous for its intended purpose.

**Environmental control**

During the entire filling process, the glove box was continuously monitored for oxygen content and hygrometry. Measurements were carried out at the beginning and end of each half day session. Values recorded did not significantly deviate from settings.

**Stability studies on the product in the final container**

An accelerated degradation study was carried out at the EDQM by storing vials of the candidate batch of the second IS for gramicidin at +20°C, +37°C and + 45°C in several climatic chambers (Binder, KBF 720 model).
Accelerated Degradation Assessed by Microbiological Assay

The potencies of these vials were estimated as the relative potencies against ampoules kept at –20°C. Two vials were analysed by two independent assays for each temperature. The data are presented in Annex 1 in both tabular and graphic format after one, three and six months of storage. In addition, a vial stored at -80°C was also used to estimate the potency of vials stored at -20°C to generate some baseline data for future monitoring purposes. No decrease in potency of the vials stored at -20°C was observed in this latter test.

Assuming that the expected recovery should be 100% in the absence of any degradation, all these values were within the ±6% acceptance criterion set to account for the variability of the analytical method based on the long history of monitoring data collected at the EDQM for gramicidin. However samples stored for a longer period at each of the elevated temperatures appeared to exhibit a systematic lower potency (compare curve in Annex 1 at 3 and 6 months respectively).

Extrapolation by means of a model based on the Arrhenius equation [2] shows that no predictable loss of potency (less than 0.1%) is expected over a period of 10 years when stored at -20 °C.

From these data it is anticipated that the stability of the second International Standard for gramicidin is satisfactory.

Accelerated Degradation Assessed by Liquid Chromatography

EDQM has a long record of experience in monitoring the stability of official Ph. Eur. reference standards for antibiotics. Due to the inherent variability of the microbiological assay methods, it was decided some years ago to replace them by stability indicating methods such as reverse phase high liquid chromatography (rp-HPLC) for monitoring the stability of the Ph. Eur. reference standards. It was therefore believed to be of benefit to estimate the degradation at elevated temperature by rp-HPLC in addition to microbiological assays with the aim of collecting data for future replacement of the method.

One vial of each of the three elevated storage temperatures was analysed using the liquid chromatography analytical method described under “Test. Related substances” of the Ph. Eur. monograph “Gramicidin, 0907”.

Individual peaks were identified on each chromatogram and their contents expressed as the mean areas in per cent by normalisation calculated from triplicate injections for each vial. The data are presented in Annex 2 in both tabular and graphic format after one, three and six months of storage.

After one, three or six months of storage, no dramatic modifications were observed in the impurity profiles of samples stored at any of the elevated temperatures when compared to the chromatogram recorded with the vials stored at –20°C. Individual peak area changes were considered within method variability and thus not significant. Furthermore, the impurity profile
of the vial stored at -80°C was comparable to the impurity profiles recorded for samples stored for six months at any elevated temperature.

These results are in agreement with the results generated by the microbiological assay method and confirm the absence of any significant degradation resulting in potency loss when vials of the proposed second International Standard for gramicidin are stored at an elevated temperature for up to six months, considering the variability of the methods respectively.

Conclusion
Vials of the proposed second International Standard for gramicidin were submitted to an accelerated degradation study to predict the stability at the customary storage temperature of –20°C. The results obtained with two orthogonal analytical methods demonstrated that the vials did not exhibit any reduction in potency nor any change in the impurity profile. It is therefore concluded that the stability of the batch at –20°C is satisfactory.

Considering that the precision of the liquid chromatography method is much better than the precision of the microbiological assay, it is believed that with respect to the variability of these methods, any significant change in the impurity profile will be detected ahead of any significant loss of potency. It is therefore proposed to monitor in the future the stability of the WHO second International Standard for gramicidin on an annual basis by means of liquid chromatography and to assess the impact of any significant modification (decrease in percentage of the principal peak / increase in the level of impurity or appearance of the new impurity peak) on the potency by the microbiological assay.

The vials of the proposed second International Standard for gramicidin are stored at the EDQM which is currently in charge of the establishment and distribution of International Standards for Antibiotics. About 1,500 vials will be made available to serve as the second WHO International Standard for Gramicidin.

Upon receipt, the material should be stored at -20°C if not used immediately. It is advised that the user dissolves the powder contained in the vial into the appropriate concentration and uses the solution within one day. Solutions should always be made fresh and never stored frozen prior to use.

Collaborative study
Participants
A total of six laboratories from different countries around the world volunteered to participate in the study. Each participant is referred to in this report by an arbitrarily assigned number, not necessarily reflecting the order of listing in the Appendix.

Samples
Each laboratory was provided with:

- 3 vials of WHO 1st IS for Gramicidin (64/010), 1000 IU/mg containing approximately 55 mg of powder per ampoule (EDQM internal code: 28117)
- 7 vials of the gramicidin candidate batch containing approximately 100 mg of powder per vial (activity about 1000 IU/mg) (EDQM internal code: 32786)
Assay method and study design
The participants were asked to estimate the potency of the gramicidin candidate batch by a microbiological turbidimetric assay method using the WHO 1st IS for Gramicidin B (64/010) as reference preparation. It was requested that any analytical method used be in compliance with requirements set in regional compendia in particular with respect to method validity criteria. A total of six independent assays were to be carried out by each participant.

Prior to carrying out the study an enquiry was carried out which demonstrated that participants were going to use very similar testing procedures. Based on this enquiry, a pilot assay was performed in the EDQM laboratory in order to develop and provide details for the study protocol, taking the Ph. Eur. as the example.

Participating laboratories were requested to follow the study protocol as far as possible and according to the prescription given in the Pharmacopoeia which is their usual reference.

Results and statistical analysis
Statistical methods
The experimental data obtained in this study were analysed as parallel line assays [3], using the SAS-System [4] (GLM procedure) and CombiStats [5]. Both programmes give identical outcomes, but the output is somewhat easier to transform to tables with the SAS-System, whereas CombiStats provides a more streamlined output for individual assays.

All assays were submitted to visual inspection of the plots to check for unusual features. Validity of the assays was assessed according to the flow chart in Figure 2. In routine situations where decisions are based only on one assay or only on a few assays, the level of significance is usually taken to be P=0.05. In collaborative studies with many participants, however, a more conservative level of significance is often used. This is because the level of P=0.05 leads to about 10 per cent errors of the first kind (incorrect rejection of assays), whereas errors of the second kind (incorrect acceptance of assays) will not influence the global outcome of the study much because of the large amount of data available. Hence, the level of significance in this study is taken to be P=0.01 which would imply an expectation of about 2 per cent incorrect rejections. A slight but significant curvature was not considered reason for rejection if the mean square for quadratic regression was less than 1/100 of the mean square for linear regression and the difference between preparations was small ([6],[7]).

Whenever a laboratory performed several assays based on the same weighings, yielding several non-independent estimates of potency, a weighted mean potency of the valid sub-assays was calculated using weights proportional to the reciprocal of the variance. Only the valid assays per laboratory were combined using the same method of weighted combination, but a semi-weighted combination was used whenever the confidence intervals of the independent potency estimates did not satisfactorily overlap each other by means of a $\chi^2$ test for homogeneity (P<0.10). The estimates (one for each of the participants) were then combined into one single estimate with a 95 per cent confidence interval using the same method of semi-weighted combination.

Results
Six (6) laboratories reported results from assays. In this report they are referred to by their randomly assigned code-numbers (1 to 6), not necessarily corresponding to the order of listing in
the list of participants. Four (4) laboratories used the European Pharmacopoeia method and two (2) used another method. All participants carried out at least 6 assays as requested. Laboratory 5 carried out 7 assays but reported having problems obtaining valid assays.

A total of 33 valid assays were reported or 1559 turbidity readings.

The complete computer output of the parallel line analyses as performed at EDQM is available in PDF format to participants of the study (59 pages generated by CombiStats). A summary of the results, as generated by the SAS-System is given in Table 3 (See Annex 3 for the essential SAS-scripts used). The potency estimates and associated 95 per cent confidence intervals are shown, together with the relevant P-values of the analysis of variance. P-values below the significance level of 0.01 are printed on a grey background. The potency estimates and confidence intervals based on calculations by the participants are also listed.

A graphical representation of the potencies and confidence intervals of each individual assay is shown in Figure 3 (EDQM calculations) and in Figure 4 (Participants’ calculations). Potency estimates ranged from 965 IU/mg (Lab 1) to 1252 IU/mg (Lab 6), based on EDQM calculations.

Laboratory 1
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.155). The weighted combined estimate is 1011 IU/mg (± 2.5%).

Laboratory 2
The 6 assays were statistically valid and the potency estimates were heterogeneous (P<0.001). The semi-weighted combined estimate is 1142 IU/mg (± 2.1%).

Laboratory 3
This laboratory used 5 doses for each assay, but only the 3 middle doses were used in the calculation. The 6 assays were statistically valid and the potency estimates were heterogeneous (P=0.039). The semi-weighted combined estimate is 1059 IU/mg (± 1.1%).

Laboratory 4
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.993). The weighted combined estimate is 1053 IU/mg (± 1.9%).

Laboratory 5
This laboratory reported having problems obtaining statistically valid assays. A total of 7 assays were performed but only 3 were judged valid by the participant and therefore they initially reported only data from these 3 assays. However, upon request from the EDQM, data from the other 4 assays were also submitted. Three (3) of the 7 assays were found to be statistically valid at EDQM. This laboratory used 5 doses for each valid assay, but only the 3 middle doses were used in the calculation. The potency estimates were heterogeneous (P<0.001). The semi-weighted combined estimate is 1044 IU/mg (± 3.1%).

Laboratory 6
The 6 assays were statistically valid and the potency estimates were heterogeneous (P<0.001). The semi-weighted combined estimate is 1102 IU/mg (± 2.9%).

A histogram of all potency estimates per assay is shown in Figure 5 and a histogram of the mean results per laboratory is shown in Figure 6. The final potency estimates and confidence intervals per laboratory are summarised in Table 4 and a graphical representation is given in Figure 7.
The $\chi^2$ value for between-laboratory variation is highly-significant (P<0.001) so a semi-weighted combination was made, which yields 1068 IU/mg (± 1.7%).

**Comments from participants**
None of the participants opposed the proposal to assign a potency of 1070 IU/mg to the Second International Standard for Gramicidin (ISA_28168).

**Recommendation**
It is proposed that the Second WHO International Standard for Gramicidin (EDQM internal code ISA_28168) be assigned an antimicrobiological activity of 1070 IU per milligram of substance. The potency of this batch was estimated by the participants of the collaborative study using the first WHO International Standard for Gramicidin as the reference standard in the microbiological assay. Therefore, the continuity of the International Unit for gramicidin originating with the establishment of the first WHO International Standard for Gramicidin is maintained.

**Acknowledgements**
The organisers express their sincere thanks to all participants for their valuable contributions to this study. Special thanks go to the donator of the gramicidin drug substance, Alpharma ApS, Copenhagen (Denmark), for their greatly appreciated contribution. The study was organised by the EDQM (project code ISA004) for the WHO. Sally Woodward is acknowledged for skilful assistance.

**References**
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By alphabetical order of contact person

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### Table 3: Overview of assay results generated by the SAS System

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay</th>
<th>Calculated by participants (IU/mg)</th>
<th>Calculated at EQQM (IU/mg)</th>
<th>p-values</th>
<th>Analysis of variance</th>
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<td>95% Lower</td>
<td>Estimated</td>
<td>95% Upper</td>
<td>95% Lower</td>
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*p*-values below the significance level of 0.01 are printed on a grey background.
Table 4
Combined potency estimates per laboratory

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<th>Estimated potency</th>
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<td>3</td>
<td>1047</td>
<td>1059</td>
<td>1070</td>
</tr>
<tr>
<td>4</td>
<td>1033</td>
<td>1053</td>
<td>1072</td>
</tr>
<tr>
<td>5</td>
<td>1013</td>
<td>1044</td>
<td>1077</td>
</tr>
<tr>
<td>6</td>
<td>1071</td>
<td>1102</td>
<td>1133</td>
</tr>
<tr>
<td>Comb.</td>
<td>1050</td>
<td>1068</td>
<td>1066</td>
</tr>
</tbody>
</table>
Figure 2
Flow chart for assay validity check

START

- Significant deviations from parallelism?
  - Yes → Reject the assay
  - No → Significant deviations from linearity?
    - Yes → Reject the assay
    - No → Significant quadratic curvature?
      - Yes → Ratio MS curvature/ regression small?
        - Yes → Accept the assay
        - No → Reject the assay
      - No → Accept the assay
  - No → Difference between preparations small?
    - Yes → Accept the assay
    - No → Reject the assay
The numbers below the 95% confidence intervals are the laboratory codes. Invalidation assays are shown with an empty dot.
Figure 4 - Individual potency estimates and 95% confidence intervals per assay (calculated by participants)
Figure 5 - Histogram of final potency estimates per vial

Figure 6 - Histogram of final potency estimates per laboratory

Numbers in the boxes are the laboratory codes
Figure 7 - Potency estimates and 95% confidence intervals per laboratory (combined assays)
ANNEX 1: Accelerated Degradation, Microbiological Assay Results

Relative Potency in per cent versus samples stored at -20°C.

<table>
<thead>
<tr>
<th>Storage temperature</th>
<th>+20°C</th>
<th>+37°C</th>
<th>+45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vial 1</td>
<td>100.7</td>
<td>102.9</td>
<td>105.7</td>
</tr>
<tr>
<td>CI 95%</td>
<td>-1.9</td>
<td>+1.9</td>
<td>-2.0</td>
</tr>
<tr>
<td>Vial 2</td>
<td>103.6</td>
<td>105.2</td>
<td>103.5</td>
</tr>
<tr>
<td>CI 95%</td>
<td>-2.0</td>
<td>+1.7</td>
<td>-1.5</td>
</tr>
<tr>
<td>Mean</td>
<td>102.1</td>
<td>104.1</td>
<td>104.5</td>
</tr>
<tr>
<td>CI 95%</td>
<td>+2.4</td>
<td>+1.7</td>
<td>+2.0</td>
</tr>
</tbody>
</table>

1 month

<table>
<thead>
<tr>
<th>Storage temperature</th>
<th>+20°C</th>
<th>+37°C</th>
<th>+45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vial 1</td>
<td>102.2</td>
<td>105.0</td>
<td>102.8</td>
</tr>
<tr>
<td>CI 95%</td>
<td>-2.8</td>
<td>+2.3</td>
<td>-2.4</td>
</tr>
<tr>
<td>Vial 2</td>
<td>104.7</td>
<td>100.7</td>
<td>100.0</td>
</tr>
<tr>
<td>CI 95%</td>
<td>-1.7</td>
<td>+2.3</td>
<td>-2.4</td>
</tr>
<tr>
<td>Mean</td>
<td>104.0</td>
<td>102.8</td>
<td>101.4</td>
</tr>
<tr>
<td>CI 95%</td>
<td>+1.5</td>
<td>+3.3</td>
<td>+2.6</td>
</tr>
</tbody>
</table>

3 months

<table>
<thead>
<tr>
<th>Storage temperature</th>
<th>+20°C</th>
<th>+37°C</th>
<th>+45°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vial 1</td>
<td>103.4</td>
<td>95.7</td>
<td>96.3</td>
</tr>
<tr>
<td>CI 95%</td>
<td>-2.0</td>
<td>-4.3</td>
<td>-5.1</td>
</tr>
<tr>
<td>Vial 2</td>
<td>102.6</td>
<td>103.8</td>
<td>97.8</td>
</tr>
<tr>
<td>CI 95%</td>
<td>-2.4</td>
<td>-4.3</td>
<td>-5.1</td>
</tr>
<tr>
<td>Mean</td>
<td>103.0</td>
<td>99.2</td>
<td>96.7</td>
</tr>
<tr>
<td>CI 95%</td>
<td>-1.5</td>
<td>-6.0</td>
<td>-2.5</td>
</tr>
</tbody>
</table>

6 months

Mean: geometric mean
CI: confidence interval \(P=0.95\)
### ANNEX 2: Accelerated Degradation, Liquid Chromatography Results

Mean Impurity Peak Areas in Per Cent

|                | 28'6 | 31'4 | 33'2 | 40'1 | 41'0 | 44'0 | 49'1 | 55'6 | 72'7 | 78'0 | 87'0 | 16'0 | 20'0 | 21'2 | 22'0 | 23'4 | 24'5 | 28'2 | 29'0 | 37'9 | 40'0 | 41'1 | 44'0 | 49'0 | 55'0 | 72'0 | 78'0 | 87'0 | 16'0 | 20'0 | 21'2 | 22'0 | 23'4 | 24'5 |
|----------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| **Peaks**      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |      |

*After 3 months*:
- Peak 1: Gramicidin C1
- Peak 2: Impurity A
- Peak 3: Impurity D
- Peak 4: Impurity B

*After 6 months*:
- Peak 1: Gramicidin C1
- Peak 2: Impurity A
- Peak 3: Impurity D
- Peak 4: Impurity B

*After 12 months*:
- Peak 1: Gramicidin C1
- Peak 2: Impurity A
- Peak 3: Impurity D
- Peak 4: Impurity B
ANNEX 2 continued

![Impurity Profiles after 1 Month](image-url)
ANNEX 2 continued

![Impurity Profiles after 3 Months](image)
ANNEX 2 continued

Impurity Profiles after 6 Months

-80°C
-20°C
+20°C
+37°C
+45°C
ANNEX 3 – SAS-Script used for the calculations

/* This is the essential script to perform the analysis of variance. It expects a SAS-dataset “gramicidin” with the following fields:
prep: 1 for standard, 2 for test.
dose: on log-scale with the primary dose set to 0.
code: copy of dose.
row: indicates the row in Latin square designs.
block: the column in Latin square designs or the block in randomized block designs.
block and/or row are set to 1 if not applicable for their respective designs.
obs: the value of the observation (possibly transformed).
*/
ods select none;
proc glm data=gramicidin;
   /* Perform the Anova by progressively relaxing model assumptions */
   class prep code block row;
   model obs=block row prep dose code*dose*prep / ss1;
   ods output OverallAnova=OverallAnova ModelAnova=ModelAnova;

data Anova(keep=source df ss ms FValue probC);
   /* Non-linearity has to be calculated in a separate datastep */
   retain dfLin ssLin;
   set OverallAnova;
   if df>0 then output;
   if Source='dose*dose' then do; dfLin=9; ssLin=ss; end;
   if Source='prep*code' then do; dfLin=dfLin+df; ssLin=ssLin+ss; end;
   if Source='Error' then do;
      Source='Non-linearity'; FValue=(ssLin/dfLin)/ms; ProbF=1-Prob(FValue,dfLin,df);
      ss=ssLin; df=dfLin; ss=ss/df;
   if df>0 then output;
   end;
ods select none;
proc print data=Anova noobs;
run;

/* This is the essential script to perform the potency calculations. It expects a SAS-dataset “info” with the following fields:
   Assigned: The assigned potency of the standard
   mgT: weight taken of the Standard
   mT: Dilution used to prepare the primary dose of the Test.
   mg7: Dilution used to prepare the primary dose of the Test.
*/
ods select none;
proc glm data=gramicidin;
   /* Fit the parallel line model and output the parameter estimates and covariance matrix */
   class block row;
   model obs=prep dose block row / inverse solution;
   ods output InvXFX=CovB ParameterEstimates=ParmEst;

data Estimate(keep=Low Est High);
   /* calculate the relative potency (m) */
   set ParmEst; where Parameter='prep'; m=Estimate;
   set ParmEst; where Parameter='dose'; b=Estimate;
   m=mean(m);
   /* Use Fieller's theorem to compute the confidence limits */
   set CovB; where Parameter='prep'; v1=b; set CovB; where Parameter='dose'; v2=b;
   v12=m*v1; v22=m*v2;
   t=tinv(0.975,df); s=sqrt(ms);
   g=abs(t*b-v22)/(s*b);
   root=v1-v2*m+v12+v22-g*(v12+v22)/v22;
   ml=(m-g*v12/v22-t/s)b*sqrt(root)/(1-g);
   m=ml;
   /* Transform the relative potency to IU by correcting for the pre-dilutions */
   set info; Correction=Assigned*mg7/mgT*mg7/mT;
   Low=Correction*exp(ml); Est=Correction*exp(m); High=Correction*exp(mU);
   output;
ods select all;
proc print data=Estimate noobs;
run;
Annex 4: Safety Data Sheet and Leaflet

SAFETY DATA SHEET
according to Directive 67/548/EC and Regulation 1907/2006/EC as amended

1. IDENTIFICATION OF THE SUBSTANCE AND OF THE COMPANY

GRAMICIDIN
European Directorate for the Quality of Medicines & HealthCare
7, Allée Kastner CS 30026, F-67081 Strasbourg (France)
Tel. +33 (0)3 88 41 20 35  Fax. +33 (0)3 88 41 27 71
For any question: www.edqm.eu (HelpDesk)

Catalogue code: ISA_28168
Use: For laboratory tests and assays only.

2. HAZARDS IDENTIFICATION

Prolonged or repeated exposure may cause allergic reactions in certain individuals (antibiotic).

3. COMPOSITION/INFORMATION ON INGREDIENTS

Gramicidin consists of a family of antimicrobial linear polypeptides, usually obtained by extraction from tyrothricin, the complex isolated from the fermentation broth of *Brevibacillus brevis* Dubos. The main component is gramicidin A1, together with gramicidins A2, B1, C1 and C2 in particular.

CAS: 1405-97-6  EC No: 215-790-4  EC Index: None.
NB: Antibiotic/antifungal.

4. FIRST AID MEASURES

Inhalation: Remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen.
Skin contact: Immediately flush skin with soap and copious amounts of water for at least 15 minutes while removing contaminated clothing and shoes. Wash contaminated clothing before reuse.
Eyes contact: Immediately flush eyes with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating the eyelids with fingers.
Ingestion: Wash out mouth with water provided person is conscious.

In case of reactions described in hazards identification or other severe, immediate or persisting symptoms seek medical advice and call the nearest poison centre. Show the label and this safety data sheet.

5. FIRE FIGHTING MEASURES

Extinguishing media: Carbon dioxide, dry chemical powder or appropriate foam. Water spray.
This substance may form dust and is sensitive to ignition.
Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes. Emits toxic fumes under fire conditions.
Dangerous combustion products: CO₂, NOₓ and other noxious gases or vapours in case of incomplete combustion.

6. ACCIDENTAL RELEASE MEASURES

Switch off electrical equipment and any other sources of ignition. Evacuate area. Wear self-contained breathing apparatus, rubber boots and heavy rubber gloves. Sweep up, place in a bag and hold for waste disposal. Avoid raising dust. Ventilate area and wash spill site after material pick up is complete.

GRAMICIDIN  REVISION 01  30/07/2008  1/3
7. HANDLING AND STORAGE

Ensure adequate ventilation.
Handling: Do not breathe dust. Do not get in eyes, on skin, on clothing. Avoid prolonged or repeated exposure. Wash thoroughly after handling.
Storage: Keep unopened in the original container at -20 °C. Protect from humidity and light.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Handle small quantities in fume hood. Safety shower and eye bath required.
Respiratory protection: Protection mask (P1)
Hand protection: Compatible chemical-resistant gloves
Eye protection: Chemical safety goggles

9. PHYSICAL AND CHEMICALS PROPERTIES

Appearance: White or almost white, crystalline powder, slightly hygroscopic.
Solubility: Practically insoluble in water, soluble in methanol, sparingly soluble in alcohol.
Melting point: About 230 °C
Flash point: Information not available.
pH: Information not available.
Log Pow: Information not available.

10. STABILITY AND REACTIVITY

Stability: Stable under recommended conditions of use.
Conditions to avoid: Avoid humidity, heat and ignition.
Materials to avoid: Not known.
Dangerous decomposition products: CO₂, NOₓ and other substances in case of incomplete decomposition. Hazardous polymerisation will not occur.

11. TOXICOLOGICAL INFORMATION

RTECS No: MD8225000
See actual entry in RTECS for complete information.
LD₅₀ (oral-rat): Information not available.
LC₅₀ (inhalation-rat): Information not available.
LC₅₀ (skin-rat): Information not available.
LD₅₀ (iv-mouse): 1.5 mg/kg
Prolonged or repeated exposure may cause allergic reactions in certain individuals.

Carcinogenicity, Mutagenicity, Reproductive toxicity and Sensitisation: None identified.

12. ECOLOGICAL INFORMATION

Toxicity:
LC₅₀ (fish-96h): Information not available.
EC₅₀ (daphnies-48h): Information not available.
IC₅₀ (algae-72h): Information not available.

Biodegradation: Information not available.

Bioaccumulation and mobility:
Log Pow: Information not available.
BCF: Information not available.

13. DISPOSAL CONSIDERATIONS

Incinerate in an approved facility.
Observe all federal state and local environmental regulations.
14. TRANSPORT INFORMATION

<table>
<thead>
<tr>
<th>Transport regulation</th>
<th>UN Classification</th>
</tr>
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<tbody>
<tr>
<td>IATA/ICAO (air)</td>
<td>Not classified</td>
</tr>
<tr>
<td>ADR/RID (road/railway)</td>
<td>Not classified</td>
</tr>
<tr>
<td>IMDG (sea)</td>
<td>Not classified</td>
</tr>
</tbody>
</table>

Marine pollutant: Not classified

15. REGULATORY INFORMATION

S phrases:
S22 Do not breathe dust.
S24/25 Avoid contact with skin and eyes.

16. OTHER INFORMATION

Revision for update:
Revision according to EC Regulation 1907/2006/EC and new catalogue code number.

R36/37/38 Irritating to eyes, respiratory system and skin.

Warning! Reference Standards - important notice.

Information applies only to the International Standard for its intended use. The EDQM staff assembles the MSDSs by using the information available at the time from sources considered reliable, such as, the European Chemical Substances Information System, approved Summaries of Product Characteristics, RTECS and the MSDS of the suppliers, manufacturers or importers. The EDQM does not independently verify the information. The accuracy of the information cannot therefore be guaranteed, nor does it constitute any expression of opinion by the EDQM concerning the substance/preparation. This information is accordingly not to be regarded as a representation or statement concerning the quality or safety of the substance, the presence of any defect in it, or its fitness for any particular purpose except that of use as an International Standard by professional persons having technical skill and at their own discretion and risk.

The downstream users have the responsibility to manage the risks arising from their use of the International Standards and for use of any information provided in this SDS.

People working with reference material from biological origin (human or animal) should apply State-of-the-art precautions.
The 2nd International Standard for Gramicidin

1. The Standard

The 2nd International Standard (IS) for Gramicidin (ISA_28168) consists of vials of approximately 100 mg of Gramicidin. This preparation was established as the 2nd IS for Gramicidin by the Expert Committee on Biological Standardization of the World Health Organisation in 2008.

2. Biological Activity

The standard was calibrated in an international collaborative study involving 6 laboratories from different countries, against the 1st IS for Gramicidin.

The assigned potency is 1070 IU/mg for the 2nd IS for Gramicidin.

3. Use of the Standard

Dissolve an appropriate amount of powder with a suitable amount of solvent using gentle shaking. Transfer the solution to a plastic tube and keep at room temperature during the assay. The solution should be used as soon as possible and should be kept at 25°C maximum during assays. Unused material must be discarded and not frozen for later use. Unopened vials should be stored at -20°C.

The powder in the vial is hygroscopic. Use appropriate measures to avoid water uptake during weighing.

4. Stability

Accelerated degradation studies have shown that the standard is stable when stored in unopened vials at -20°C, with no predictable loss of potency over a period of 36 months. It is therefore recommended that the unopened vials should be stored at -20°C or below until immediately before use.

5. References

Collaborative Study for the Establishment of the Second International Standard for Gramicidin B, WHO/BS/08.xxx

6. Caution

This material is not for administration to humans. Safety Data Sheet is available on the EDQM website (www.edqm.eu) or on request.
7. Citation

In all publications (or data sheets for kits) in which this preparation is used as an assay calibrant, it is important that the title of the preparation, code and the name and addresses of EDQM are cited correctly.

8. Product liability

The Council of Europe accordingly makes no representation, contractual statement, or expression of opinion concerning the quality or safety of any item supplied, the presence of any defect in it, or its fitness for any particular purpose. The product must be handled by professional persons having technical skill and at their own discretion and risk. It is for the purchasers of any such item who are responsible for persons in a workplace to determine independently the risks associated with the item according to the conditions of use and to take appropriate safety measures, including provision of appropriate information to persons working with the substance. Any liability of the Council of Europe for injury, loss or damage arising from the supply or use of any such item is in any event hereby excluded to the fullest extent permitted by law; in particular, no liability is accepted for loss of profits or indirect or consequential loss.

Disputes

In accordance with the provisions of article 21 of the General Agreement on the Privileges and Immunities of the Council of Europe, all disputes between the Council of Europe (EDQM) and the customer as regards the application of this contract shall be submitted, if a mutual agreement cannot be reached between the parties, to arbitration as laid down in Order No. 481 of the Secretary General, approved by the Committee of Ministers.