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Collaborative Study for the Establishment of the Third International Standard for Neomycin

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Note:
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Summary

An international collaborative study was organised to establish the third World Health Organization (WHO) International Standard (IS) for neomycin. The report presents this study in which 10 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 second IS for neomycin 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 for Biological Standardization (ECBS). It is proposed that the WHO Third International Standard for Neomycin be assigned an antimicrobiological activity of 19 050 IU per vial.

Introduction

Neomycin is an aminoglycoside antibiotic found in many topical medications or given orally mainly prior to gastrointestinal surgery. It is not absorbed from the gastrointestinal tract and has shown an excellent activity against Gram-negative bacteria, and partially against Gram-positive bacteria. By killing bacteria in the intestinal tract, it keeps ammonia levels low and thus contributes to the prevention of hepatic encephalopathy. Due to its severe nephrotoxicity, neomycin is never administered intravenously. Aminoglycosides are known for their ability to bind to duplex RNA with high affinity and more recently have been shown to stabilize triplex DNA structure.

The second IS for neomycin was established by the WHO in 1973 on the basis of an international collaborative study [1]. It was assigned with a potency of 775 International Units per mg (IU/mg), each ampoule containing approximately 50 mg.

As stocks of the 2nd IS were becoming exhausted, the European Directorate for the Quality of Medicines & HealthCare (EDQM), the WHO collaborative centre for antibiotics, 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 consisting of neomycin of pharmaceutical grade was kindly donated to the EDQM by Upjohn. This material was appropriate for therapeutic use and declared to comply with the quality standards of the European Pharmacopoeia (Ph. Eur.) monograph “Neomycin sulfate, 0197”. A certificate of analysis was provided in the batch documentation.

Production of the WHO third IS for neomycin candidate batch

Due to the potential hygroscopic character of the neomycin powder, it was decided to distribute the standard as a freeze-dried preparation rather than a powder fill as was the case with the previous IS. The parameters of the freeze-drying process were derived from information obtained from a company manufacturing freeze-dried dosage forms.
All powder weighing was performed in a glove box under controlled atmosphere by use of argon gas. Several vials containing precisely weighed amounts were prepared concomitantly to enable further testing of the bulk powder and to prepare the solution to be freeze-dried.

The bottle containing neomycin sulfate bulk was allowed to equilibrate at room temperature and was subsequently submitted to homogenisation in a Turbula mixer. Formulation: 25.46 g of neomycin bulk were dissolved in 1000.1 g of purified water and stirred until complete dissolution. The final concentration of the solution was 25.46 mg neomycin bulk per gram of solution.

Filling: The solution was filled into 9.0 mL amber glass vials. The theoretical filling weight was 1.0 g.

Control of filling weight: 20 vials were randomly sampled across the lot. Results were as follows: mean filling weight: 1.0082 g; RSD: 0.07%. The filling was considered homogeneous.

Lyophilisation: The vials were placed onto 4 trays and underwent lyophilisation. The complete process was registered under the batch number Fab 11/12-53. A total of 980 vials were produced.

Selection of a batch suitable as “reference standard” for monitoring purposes
WHO IS are primary reference materials and as such they 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 is assessed by means of accelerated degradation studies. Nevertheless, it was decided to randomly sample 30 vials from the batch of the third WHO International Standard for Neomycin and to store them at –80°C. The intention is to use them, 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. Vials stored at -80°C were registered under EDQM internal number 49163.

Quality control on bulk and final batch

Visual appearance of final vials
Vials were randomly sampled from the freeze-dried batch and inspected visually. The appearance of the cakes was judged satisfactory.

Residual water content
It was decided to estimate the residual water content in 6 vials randomly sampled from the batch. The determination of water was performed as described in the Ph. Eur. general chapter “2.5.32. Water: Micro determination”.
The individual water content estimates of all vials were below the quantification limit of 150 micrograms corresponding to about 0.6 per cent (w/w). The residual water content was therefore not believed to have an adverse effect on the stability of the batch upon storage.

Homogeneity of neomycin content in final vials
The homogeneity of the neomycin content in 10 vials randomly sampled across the batch was assessed by using the liquid chromatography (LC) method described in the Ph. Eur. monograph “Neomycin sulfate, 0197” under “Test. Related substances”.
The entire content of each vial was reconstituted in a final volume of 50.0 mL with mobile phase A after thorough rinsing to ensure quantitative transfer. Ten microliters of each solution were injected in triplicate. Almost identical impurity profiles were recorded with all samples and inter-vial variability did not appear to be visually significant. The peaks corresponding to neomycin were identified on each chromatogram and peak areas were used to calculate the mean and RSD. A value of 2.03 % was obtained for the latter and it was considered that the homogeneity of the neomycin content of the batch was satisfactory.

**Stability studies on the product in the final container**
An accelerated degradation study was carried out at the EDQM by storing freeze-dried vials of the candidate batch of the third WHO IS for neomycin at +20°C, +37°C and +45°C in climatic chambers (Binder, KBF 720 model) for 1, 3 and 6 months respectively. Due to severe time constraints, the analysis at the 6 month time point could not yet be completed and results will be communicated at a later date.

**Accelerated Degradation Assessed by Microbiological Assay**
The potencies of these vials were estimated as the relative potencies against vials of the same batch kept at -20°C. Two vials were analysed by two independent assays for each temperature using the diffusion method. The data are presented in Annex 1 in tabular and graphic format after one and three months of storage respectively.

Assuming that the expected recovery should be 100% in the absence of any degradation, all the values were within the ±5% acceptance criterion set to account for the variability of the analytical method based on the long history of monitoring data collected at the EDQM. It is therefore considered that the apparent drop in potency, if any, is only marginal even after 3 months storage at elevated temperatures.

From these data it is anticipated that the stability of the third WHO IS for neomycin is satisfactory at the customary storage temperature of -20°C.

**Accelerated Degradation Assessed by Liquid Chromatography**
EDQM has a long record of experience in monitoring the stability of official European Pharmacopoeia (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 reversed phase liquid chromatography (LC) 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 LC in addition to microbiological assays.

Two vials from each of the three storage temperatures were analysed using the liquid chromatography analytical method described under “Test. Related substances” of the Ph. Eur. monograph “Neomycin sulfate, 0197”.

Individual peaks were identified on each chromatogram and their contents were expressed as the mean areas by normalisation calculated from triplicate injections for each vial. The data are presented in tabular format in Annexes 2 and 3 after one and three months’ storage respectively and in graphic format in Annex 4.

The data indicate that the impurity contents of samples stored at any of the elevated temperatures for either one or three months were almost identical since their chromatographic profiles were highly similar. Individual peak area differences might be considered to reflect method variability rather than a change resulting from degradation.
These results are in good 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 third International Standard for neomycin are stored at elevated temperature for up to three months.

**Conclusions from Accelerated Degradation**

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 when stored at elevated temperature after 1 or 3 months. From the data obtained so far it is not foreseen that vials analysed after the 6 month time period will exhibit a dramatic reduction in either potency or neomycin content. It is therefore believed that the stability of the batch at the customary storage temperature of −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 batch 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 new impurity peak) on the potency by the microbiological assay.

The vials of the proposed third International Standard for neomycin are stored at the EDQM which is currently in charge of the establishment and distribution of the WHO International Standards for Antibiotics.

Upon receipt, the vials should be stored at -20°C if not used immediately. It is advised that the user dissolves the freeze-dried cake contained in the vial to generate the appropriate concentration. This solution may be used within three days if stored at 4°C should non-valid test results trigger repeat testing. No attempt to weigh out the freeze-dried cake should be made. Solutions should always be made fresh and never stored frozen prior to use.

**Collaborative study**

**Participants**

A total of 10 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 2nd IS for Neomycin (72/406), 775 IU per mg containing approximately 50 mg of powder per ampoule (EDQM internal code: 39915)
- 7 vials of the Neomycin 3rd IS candidate batch, activity about 19000 IU per vial (EDQM internal code: 46707)

**Assay method and study design**

The participants were instructed to exercise extreme care while dissolving the freeze-dried cake of the candidate batch.
They were asked to estimate the potency of the neomycin 3rd IS candidate batch by the microbiological activity on target micro-organisms. The current WHO 2nd IS for Neomycin was used as reference standard.

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 design as far as possible and according to the prescription given in the compendia which is their usual reference.

**Results and statistical analysis**

**Statistical methods**

The experimental data obtained in this study were analysed as parallel line assays [2], using the SAS-System [3] (GLM procedure) and CombiStats [4]. Both programs 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 1. In routine situations where decisions are based on only one assay or only 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 [5,6].

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. 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**

Ten (10) laboratories reported results from assays. One laboratory submitted 2 sets of assays performed by 2 different operators and are treated in this report as if they are from 2 different
laboratories. Laboratories are referred to by their randomly assigned code-numbers (1 to 11), not necessarily corresponding with the order of listing in the list of participants. All participants carried out at least 6 assays as requested. Only the method by diffusion was performed although the protocol also allowed the use of the turbidimetric method. Laboratory 5 submitted data from 5 assays carried out in triplicate resulting in a total of 15 sub-assays. This laboratory used only 2 doses per preparation so it was not possible to check for non-linearity of the dose-response curves. Laboratory 6 used a design in which the standard was tested at several dose levels, but the test preparation at only 1 dose. It was therefore not possible to check for parallelism. For Laboratory 8 it was necessary to use a logarithmic transformation of the responses in order to improve linearity. Laboratories 10 and 11 repeated some assays resulting in a total of 9 assays for Laboratory 10 and 7 assays for Laboratory 11. For the calculations, all sub-assays were analyzed as individual assays after which they were combined into one potency estimate per vial. If all sub-assays are counted as individual assays a total of 79 assays were reported or 4161 zone-diameter readings.

The complete computer output of the parallel line analyses as performed at the EDQM is available in PDF format to participants of the study (158 pages generated by CombiStats). A summary of the results, as generated by the SAS-System is given in Tables 1.1 and 1.2 (See Annex 1 for the essential SAS-scripts used). Shown are the potency estimates and associated 95 per cent confidence intervals, together with the relevant P-values. P-values below the significance level of 0.01 are printed on a grey background. The confidence intervals based on calculations by the participants are also listed.

A graphical representation of the confidence intervals of each individual (sub)-assay is shown in Figure 2 (EDQM calculations) and in Figure 3 (Participants’ calculations). Potency estimates from valid assays ranged from 16334 IU/vial (Lab 2) to 20874 IU/vial (Lab 11). If the results from Laboratory 2 are disregarded the range is from 17278 IU/vial to 20874 (both extremes by Lab 11). Combined potency estimates are shown in Table 2.

**Laboratory 1**
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.964). The weighted combined estimate is 18528 IU/vial (±2.3%).

**Laboratory 2**
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.484). The weighted combined estimate is 16667 IU/vial (±1.3%).

**Laboratory 3**
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.775). The weighted combined estimate is 18892 IU/vial (±1.4%).

**Laboratory 4**
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.100). The weighted combined estimate is 19549 IU/vial (±1.5%).

**Laboratory 5**
The 2nd replicate of assay 5 had to be declared invalid due to significant deviations from parallelism. The other 14 sub-assays were statistically valid and were first combined to obtain 5 potency estimates (1 for each vial). The resulting 5 potency estimates were heterogeneous (P<0.001). The semi-weighted combined estimate is 18414 IU/vial (±1.0%).
Laboratory 6
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.992).
The weighted combined estimate is 19502 IU/vial (±1.9%).

Laboratory 7
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.814).
The weighted combined estimate is 19269 IU/vial (±1.6%).

Laboratory 8
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.629).
The weighted combined estimate is 19409 IU/vial (±0.7%).

Laboratory 9
The 6 assays were statistically valid and the potency estimates were homogeneous (P=0.784).
The weighted combined estimate is 19209 IU/vial (±5.0%).

Laboratory 10
The 1st replicate of assay 1 contained too many outliers to be considered valid and was excluded for that reason. The other 8 sub-assays were statistically valid and were first combined to obtain 6 potency estimates (1 for each vial). The resulting 6 potency estimates were heterogeneous (P<0.001). The semi-weighted combined estimate is 18748 IU/vial (±1.8%).

Laboratory 11
The 7 sub-assays were statistically valid and were first combined to obtain 6 potency estimates (1 for each vial). The resulting 6 potency estimates were heterogeneous (P<0.001). The semi-weighted combined estimate is 18965 IU/vial (±2.6%).

A histogram of all potency estimates per assay is shown in Figure 4 and a histogram of the mean results per laboratory is shown in Figure 5. The final confidence intervals per laboratory are summarised in Table 2 and a graphical representation is given in Figure 6. The χ² value for between-laboratory homogeneity is highly significant (P<0.001) so a semi-weighted combination was made which yields 18773 IU/vial. However, the results from Laboratory 2 deviate by 12.5% from the overall mean of the other laboratories. This laboratory should therefore be considered an outlier. They reported difficulties in opening 2 of the 3 vials of the WHO 2nd IS for neomycin serving as reference standard and consequently used only one single weighing of the third vial for all six assays. Results of this laboratory should therefore be considered with caution and were excluded from the pool of data. The semi-weighted combination excluding this laboratory is 19048 IU/vial with 95% confidence limits of 18920 to 19177 IU/vial (which is ±0.7%).

Comments from Participants
None of the participants of the study opposed the conclusions of the report.

Recommendation
The proposed candidate batch is suitable for its intended purpose. It is proposed that the WHO Third International Standard for Neomycin be assigned an antimicrobiological activity of 19 050 IU per vial.

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 neomycin drug substance, Upjohn. The study was organised by the EDQM (project code ISA011) as the WHO custodian centre for antibiotics. Sally Woodward is acknowledged for skilful assistance.
References

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By alphabetical order of contact person

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S. TYSKI
National Medicines Institute, Warsaw - Poland
Figure 1
Flow chart for assay validity check

START

Significant deviations from parallelism?

Yes → Reject the assay

No

Significant deviations from linearity?

Yes → Accept the assay

No

Significant lack of quadratic fit?

Yes → Reject the assay

No

Significant quadratic curvature?

Yes → Accept the assay

No

Ratio MS curvature/ regression small?

Yes → Accept the assay

No → Reject the assay

Difference between preparations small?

Yes → Accept the assay

No → Reject the assay
### Table 1.1

Overview of assay results generated by the SAS System

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay</th>
<th>Calculated by participants (L(U)/VI)</th>
<th>Calculated at EDOM (L(U)/VI)</th>
<th>p-Values Analysis of variance</th>
<th>Quad. regr./ Lin. regr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Estimated Potency</td>
<td>95% Confidence Limits</td>
<td>Estimated Potency</td>
<td>95% Confidence Limits</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>18645 (94.0% - 106.3%)</td>
<td>18645 (94.0% - 106.3%)</td>
<td>0.899</td>
<td>0.068</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>18479 (93.0% - 107.5%)</td>
<td>18479 (93.0% - 107.5%)</td>
<td>0.892</td>
<td>0.053</td>
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<tr>
<td></td>
<td>3</td>
<td>18000 (94.8% - 105.5%)</td>
<td>18900 (94.8% - 105.5%)</td>
<td>0.826</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>18506 (95.2% - 105.1%)</td>
<td>18506 (95.2% - 105.1%)</td>
<td>0.377</td>
<td>0.052</td>
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<tr>
<td></td>
<td>5</td>
<td>18400 (94.1% - 106.2%)</td>
<td>18400 (94.1% - 106.2%)</td>
<td>0.348</td>
<td>0.056</td>
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<tr>
<td></td>
<td>6</td>
<td>18264 (95.1% - 105.1%)</td>
<td>18264 (95.1% - 105.1%)</td>
<td>0.240</td>
<td>0.026</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>16334 (96.6% - 103.4%)</td>
<td>16334 (96.6% - 103.4%)</td>
<td>0.237</td>
<td>0.053</td>
</tr>
<tr>
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<td>16752 (96.4% - 103.7%)</td>
<td>16752 (96.4% - 103.7%)</td>
<td>0.116</td>
<td>0.088</td>
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<tr>
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<tr>
<td></td>
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<td>16996 (96.8% - 103.2%)</td>
<td>16996 (96.6% - 103.2%)</td>
<td>0.526</td>
<td>0.059</td>
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<td>5</td>
<td>16724 (97.0% - 103.0%)</td>
<td>16724 (97.0% - 103.0%)</td>
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<tr>
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<td>6</td>
<td>16407 (98.3% - 103.3%)</td>
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<td>0.557</td>
<td>0.331</td>
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<tr>
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<td>1</td>
<td>18491 (96.3% - 103.9%)</td>
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<td>0.213</td>
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<tr>
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<td>0.101</td>
<td>0.375</td>
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<td>18662 (96.1% - 104.1%)</td>
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<td>19055 (96.5% - 103.6%)</td>
<td>0.357</td>
<td>0.354</td>
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<td>18510 (95.6% - 104.6%)</td>
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<td>0.210</td>
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<tr>
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<td>19277 (97.4% - 102.7%)</td>
<td>19232 (97.4% - 102.7%)</td>
<td>0.194</td>
<td>0.205</td>
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<tr>
<td></td>
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<td>18477 (94.8% - 105.4%)</td>
<td>0.584</td>
<td>0.336</td>
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<td></td>
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<td>18712 (95.6% - 104.6%)</td>
<td>18956 (95.4% - 104.8%)</td>
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<td>0.083</td>
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<td>18394 (94.7% - 105.6%)</td>
<td>19664 (94.4% - 105.9%)</td>
<td>0.574</td>
<td>0.108</td>
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<tr>
<td></td>
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<td>19225 (97.2% - 102.9%)</td>
<td>19800 (97.1% - 103.0%)</td>
<td>0.998</td>
<td>0.049</td>
</tr>
</tbody>
</table>

- P-values below the significance level of 0.01 are printed on a grey background. n.r. = not reported. n.a. = not applicable.
### Table 1.2
Overview of assay results generated by the SAS System

<table>
<thead>
<tr>
<th>Lab</th>
<th>Assay</th>
<th>Calculated by participants (IU/Well)</th>
<th>Calculated at EDOM (IU/Well)</th>
<th>p-Values Analysis of variance</th>
<th>Quad. regr./Lin. regr.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Estimated Potency</td>
<td>95% Confidence Limits</td>
<td>Estimated Potency</td>
<td>95% Confidence Limits</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>19480 (99.0% - 101.0%)</td>
<td>19499 (98.5% - 101.5%)</td>
<td>0.795</td>
<td>0.110</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19578 (98.6% - 101.4%)</td>
<td>19526 (98.3% - 101.8%)</td>
<td>0.402</td>
<td>0.371</td>
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<tr>
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<td>19263 (98.9% - 101.2%)</td>
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<td>0.213</td>
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<tr>
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<td>4</td>
<td>19522 (98.6% - 101.5%)</td>
<td>19539 (98.1% - 101.9%)</td>
<td>0.724</td>
<td>0.084</td>
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<td>19344 (98.1% - 102.0%)</td>
<td>19312 (97.8% - 102.3%)</td>
<td>0.626</td>
<td>0.009</td>
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<tr>
<td></td>
<td>6</td>
<td>19461 (98.2% - 101.8%)</td>
<td>19477 (97.9% - 102.1%)</td>
<td>0.830</td>
<td>0.293</td>
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<td>9</td>
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<td>19184 (82.7% - 120.9%)</td>
<td>19179 (82.7% - 120.9%)</td>
<td>0.703</td>
<td>0.119</td>
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<td>2</td>
<td>19988 (98.4% - 113.0%)</td>
<td>19483 (98.4% - 113.0%)</td>
<td>0.974</td>
<td>0.841</td>
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<td>18121 (84.9% - 117.6%)</td>
<td>18117 (84.9% - 117.6%)</td>
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<td>0.956</td>
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<td>18559 (90.4% - 110.5%)</td>
<td>18554 (90.4% - 110.5%)</td>
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<td>0.191</td>
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<td>20428 (90.0% - 111.3%)</td>
<td>20423 (90.0% - 111.3%)</td>
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<td>0.689</td>
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<td>6</td>
<td>19126 (87.8% - 114.0%)</td>
<td>19121 (87.8% - 114.0%)</td>
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<td>0.360</td>
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<td>1.1</td>
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<td>18450 (95.4% - 104.6%)</td>
<td>18445 (95.9% - 104.2%)</td>
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<td>0.096</td>
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<td>17643 (95.4% - 104.5%)</td>
<td>17331 (95.2% - 104.6%)</td>
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<td>19154 (97.2% - 102.9%)</td>
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<td>0.077</td>
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<td>18140 (97.4% - 102.7%)</td>
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<td>19504 (97.4% - 102.7%)</td>
<td>19645 (97.5% - 102.6%)</td>
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<td>0.031</td>
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<td>18882 (98.0% - 102.0%)</td>
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<td>0.037</td>
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<td>17674 (96.9% - 103.1%)</td>
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<td>19665 (98.6% - 101.4%)</td>
<td>19592 (98.7% - 101.3%)</td>
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<td>20378 (95.5% - 104.8%)</td>
<td>19837 (97.5% - 102.6%)</td>
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<td>0.844</td>
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<td>0.049</td>
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<td>18200 (96.3% - 103.9%)</td>
<td>0.172</td>
<td>0.042</td>
</tr>
</tbody>
</table>

*P*-values below the significance level of 0.01 are printed on a grey background. n.r. = not reported. n.a. = not applicable.
The numbers below the 95% confidence intervals are the laboratory codes. Invalid assays are shown with an empty dot.

Figure 2

Individual potency estimates per assay and 95% confidence intervals (calculated at EDOM)
The numbers below the 95% confidence intervals are the laboratory codes. Assays considered invalid by the participating laboratories are shown with an empty dot (none in this study).

**Figure 3**

Individual potency estimates per assay and 95% confidence intervals (calculated by participants)
Figure 4 - Histogram of final potency estimates per assay

Figure 5 - Histogram of final potency estimates per laboratory

Numbers in the boxes are the laboratory codes.
### Table 2
Combined potency estimates per laboratory

<table>
<thead>
<tr>
<th>Lab</th>
<th>Final potency estimates (IU/vial)</th>
<th>95% Confidence Limits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estimated potency</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>18528</td>
<td>(97.8% - 102.3%)</td>
</tr>
<tr>
<td>2</td>
<td>16667</td>
<td>(98.7% - 101.3%)</td>
</tr>
<tr>
<td>3</td>
<td>18892</td>
<td>(98.6% - 101.4%)</td>
</tr>
<tr>
<td>4</td>
<td>19549</td>
<td>(98.5% - 101.5%)</td>
</tr>
<tr>
<td>5</td>
<td>18414</td>
<td>(99.1% - 101.0%)</td>
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<tr>
<td>6</td>
<td>19502</td>
<td>(98.1% - 102.0%)</td>
</tr>
<tr>
<td>7</td>
<td>19269</td>
<td>(98.4% - 101.6%)</td>
</tr>
<tr>
<td>8</td>
<td>19409</td>
<td>(99.4% - 100.7%)</td>
</tr>
<tr>
<td>9</td>
<td>19209</td>
<td>(95.2% - 105.1%)</td>
</tr>
<tr>
<td>10</td>
<td>18748</td>
<td>(98.2% - 101.9%)</td>
</tr>
<tr>
<td>11</td>
<td>18965</td>
<td>(97.4% - 102.6%)</td>
</tr>
<tr>
<td>Combined</td>
<td>18773</td>
<td>(99.0% - 101.0%)</td>
</tr>
<tr>
<td>Combined*</td>
<td>19048</td>
<td>(99.3% - 100.7%)</td>
</tr>
</tbody>
</table>

* Excluding Laboratory 2
ANNEX 1: Accelerated Degradation, Microbiological Assay Results

The numbers below the 95% confidence intervals are the laboratory codes.

Figure 6: Potency estimates per laboratory and 95% confidence intervals.
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>
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<td></td>
</tr>
<tr>
<td>CI 95%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1 month</td>
<td></td>
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</tr>
<tr>
<td>Vial 1</td>
<td>98.2</td>
<td>98.5</td>
<td>97.8</td>
</tr>
<tr>
<td>CI 95%</td>
<td>94.0-102.5</td>
<td>94.9-102.2</td>
<td>94.2-101.6</td>
</tr>
<tr>
<td>Vial 2</td>
<td>102.9</td>
<td>98.6</td>
<td>101.3</td>
</tr>
<tr>
<td>CI 95%</td>
<td>95.9-110.5</td>
<td>94.1-103.3</td>
<td>93.2-110.2</td>
</tr>
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<td>Mean</td>
<td>100.6</td>
<td>98.6</td>
<td>99.6</td>
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<tr>
<td>3 months</td>
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<tr>
<td>Vial 1</td>
<td>97.2</td>
<td>96.7</td>
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</tr>
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<td>CI 95%</td>
<td>93.7-100.8</td>
<td>93.0-100.6</td>
<td>94.0-100.8</td>
</tr>
<tr>
<td>Vial 2</td>
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<td>98.1</td>
<td>100</td>
</tr>
<tr>
<td>CI 95%</td>
<td>96.7-103.6</td>
<td>94.6-101.8</td>
<td>96.0-104.2</td>
</tr>
<tr>
<td>Mean</td>
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<td>98.7</td>
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</table>

Microbiological Assay Results

![Graph showing Microbiological Assay Results](image)
<table>
<thead>
<tr>
<th>Peak 16</th>
<th>Peak 15</th>
<th>Peak 14</th>
<th>Peak 13</th>
<th>Peak 12</th>
<th>Peak 11</th>
<th>Peak 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.87</td>
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<td>1.84</td>
<td>1.83</td>
<td>1.81</td>
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<td>1.44</td>
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</tr>
</tbody>
</table>

**Mean Impurity Peak Areas in Percent after one month at elevated temperatures**

**ANNEX 2: Accelerated Degradation Liquid Chromatography Results**

**Mean %**

<table>
<thead>
<tr>
<th>Peak 16</th>
<th>Peak 15</th>
<th>Peak 14</th>
<th>Peak 13</th>
<th>Peak 12</th>
<th>Peak 11</th>
<th>Peak 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3%</td>
<td>6.5%</td>
<td>5.1%</td>
<td>4.9%</td>
<td>5.3%</td>
<td>5.1%</td>
<td>4.9%</td>
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</tbody>
</table>

**Mean % of Assay**

<table>
<thead>
<tr>
<th>Peak 16</th>
<th>Peak 15</th>
<th>Peak 14</th>
<th>Peak 13</th>
<th>Peak 12</th>
<th>Peak 11</th>
<th>Peak 10</th>
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<td>95.2%</td>
<td>95.4%</td>
<td>95.1%</td>
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</table>

**Mean Impurity Peak Areas in Percent after one month at elevated temperatures**

**ANNEX 2: Accelerated Degradation Liquid Chromatography Results**

**Mean %**

<table>
<thead>
<tr>
<th>Peak 16</th>
<th>Peak 15</th>
<th>Peak 14</th>
<th>Peak 13</th>
<th>Peak 12</th>
<th>Peak 11</th>
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**Mean % of Assay**

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<thead>
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<th>Peak 15</th>
<th>Peak 14</th>
<th>Peak 13</th>
<th>Peak 12</th>
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ANNEX 3: Accelerated Degradation Liquid Chromatography Results.

Mean Peak Areas in Percent after Three Months at Elevated Temperatures.
ANNEX 4: Impurity Profiles after 1 and 3 Months at Elevated Temperatures

Individual Mean Peak Areas by Normalisation
ANNEX 5: SAS-Script used for the calculations

/* This is the essential script to perform the analysis of variance. It expects a SAS-dataset "NeoMy" 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 petri-dish in randomised 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=NeoMy;
  /* Perform the ANOVA by progressively relaxing model assumptions */
  class prep code block row;
  model obs=block row prep dose dose[prep] dose*dose code[prep] / ssl;
  ods output OverallAnova=OverallAnova ModelAnova=ModelAnova;

data Anova(keep=source df ss ms fvalue probF);
  /* Non-linearity has to be calculated in a separate datastep */
  retain dfLin ssLin;
  set ModelAnova OverallAnova;
  if df>0 then output;
  if Source='dose*dose' then do; dfLin=dfLin+df; ssLin=ssLin+ss; end;
  if Source='prep' then do; dfLin=dfLin+df; ssLin=ssLin+ss; end;
  if Source='Error' then do;
    Source='Non-linearity'; fValue=(ssLin/dfLin)/ms; ProbF=1-ProbF(fValue,dfLin,df);
    ss=ssLin; df=dfLin; ms=ss/df;
    if df>0 then output;
  end;
ods select all;
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
mgS: weight taken of the Standard
mlS: Dilution used to prepare the primary dose of the Standard
mgT: weight taken of the Test
mlT: Dilution used to prepare the primary dose of the Test.
*/
ods select none;
proc glm data=NeoMy;
  /* 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 INVX=X=COVB ParameterEstimates=ParmEst;

data Estimate(keep=Low Est High);
  /* calculate the relative potency [m] */
  set ParmEst; where Parameter='prep'; a=Estimate;
  set ParmEst; where Parameter='dose'; b=Estimate;
  m=a/b;
  /* Use Fieller's theorem to compute the confidence limits */
  set COVB; where Parameter='prep'; v11=COVB;
  set COVB; where Parameter='dose'; v12=COVB;
  set Anova; where source='Error'; t=tinv(0.975,df); s=sqrt(ms);
  g=(t+b)/b**2;
  root=v11-tr^2+2*m+2*root(v12-g\{v11-v12\}/v12),v22);
  ml=(m-g)*v12/v22-t/s^2*b**2*sqrt(root);[l-g];
  ml=(m-g)*v12/v22-t/s^2*b**2*sqrt(root);[l-g];
  /* Transform the relative potency to IU by correcting for the pre-dilutions */
  set infO; Correction=mgS/mlS*mlT/mgT;
  Low=Correction*exp(ml); Est=Correction*exp[m]; High=Correction*exp[mU];
  output;
ods select all;
proc print data=Estimate noobs;
run;
ANNEX 6: Safety Data Sheet and Leaflet

SAFETY DATA SHEET

1. IDENTIFICATION OF THE SUBSTANCE AND OF THE COMPANY

NEOMYCIN

European Directorate for the Quality of Medicines & Healthcare
European Pharmacopoeia (Ph. Eur.)
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 laboratory tests and assays only
For any question: www.edqm.eu (HelpDesk)

Catalogue code: ISA_XXXX

2. HAZARDS IDENTIFICATION

Harmful. May cause sensitisation by inhalation and skin contact. Irritating to eyes, respiratory system and skin.
Prolonged or repeated exposure may cause allergic reactions in certain individuals (antibiotic).
This substance is not classified in the table 3.1 of Annex VI of Regulation (EC) No 1272/2008.

3. COMPOSITION/INFORMATION ON INGREDIENTS

Mixture of sulphates of substances produced by the growth of certain selected strains of Streptomyces fradiae, the main component being the sulphate of 2-deoxy-4-O-(2,6-diamino-2,6-dideoxy-o-D-glucopyranosyl)-5-O-[3-O-(2,6-diamino-2,6-dideoxy-β-L-idopyranosyl)-β-D-ribofuranosyl]-D-streptamine (neomycin B).

C_{21}H_{32}N_{7}O_{12}·H_{2}SO_{4} \quad M_r \: 615 \text{ (base)} \quad CAS: 1405-10-3 \quad EC No: 215-773-1

NB: antibiotic.
Symbol: Xn.
R phrases: R63, R42/43, R36/37/38.

4. FIRST AID MEASURES

Inhalation: Remove to fresh air. If breathing becomes difficult, call a physician.
Skin contact: Flush with copious amounts of water for at least 15 minutes. Remove contaminated clothing and shoes.
Eyes contact: Call a physician. Assure adequate flushing by separating the eyelids with fingers. Flush with copious amounts of water for at least 15 minutes.
Ingestion: Call a physician. 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: Water spray. Carbon dioxide, dry chemical powder or appropriate foam.
Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes. Emits toxic fumes under fire conditions.

6. ACCIDENTAL RELEASE MEASURES

Spill should be handled by trained cleaning personnel equipped with respiratory and eye protection. Sweep up, place in a bag and hold for waste disposal. Avoid raising dust. Ventilate area and wash spill site with water after material pick up is complete.
Mechanical exhaust required.

7. HANDLING AND STORAGE

Personal protection: Wear gloves. Use in laboratory fume hood or similar equipment extracting air and/or wear suitable eye and respiratory protection.

NEOMYCIN

REVISION 03 28/06/2012 1/3
Handling: Handle with care in accordance with good occupational hygiene, safety and laboratory practises. Avoid all unnecessary exposure. Do not breathe dust. Avoid contact with eyes, skin or clothing. Avoid prolonged or repeated exposure. Wash thoroughly after handling.

Storage: Not intended for long-term storage. For quality reasons apply EDQM recommended storage conditions: Protect from light and humidity. Keep tightly closed. Keep in the original container at about -20 °C.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Respiratory protection: Protecting mask (P1).
Hand protection: Compatible chemical-resistant gloves.
Eye protection: Chemical safety goggles

9. PHYSICAL AND CHEMICALS PROPERTIES

Appearance: White or yellowish-white powder.
Solubility: Hygroscopic, very soluble in water, very slightly soluble in alcohol, practically insoluble in acetone.

10. STABILITY AND REACTIVITY

Incompatibilities: Strong oxidizing agents.
Hazardous combustion or decomposition products: COx, NOx, SOx. Hazardous polymerisation will not occur.

11. TOXICOLOGICAL INFORMATION

RTECS No: QP4375000
LD50 (oral-mouse): > 8 000 (base) mg/kg.
Laboratory experiments have shown teratogenic effects.
See actual entry in RTECS for complete information.

12. ECOLOGICAL INFORMATION

Not available.

13. DISPOSAL CONSIDERATIONS

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

14. TRANSPORT INFORMATION

Not applicable.

15. REGULATORY INFORMATION

Symbol: Xn.
R63, R42/43, R36/37/38, S22, S45, S36/37/39.

16. OTHER INFORMATION

Reason for update:
Revision according to EC Regulation.
R63 Possible risk of harm to the unborn child.
R42/43 May cause sensitization by inhalation or by skin contact
R36/37/38 Irritating to eyes, respiratory system and skin

Warning! Reference Substance - important notice
The chemical substance or biological preparation to which this Safety Data Sheet relates is supplied exclusively as a reference substance/preparation for chemical or biological test and assay purposes and on the basis of the Conditions of Supply and notes on Use of Reference Materials set out in the European Pharmacopoeia catalogue. It is to be used for no other purpose and is not for human or animal consumption. The information set out in this sheet is applicable solely to the substance/preparation when used as a European Pharmacopoeia Chemical Reference Substance (Ph. Eur. CRS) or Biological Reference Preparation (Ph. Eur. BRP) and is not intended to apply to any other use or preparation of the substance (e.g. at different concentrations, in drug dosage form or in bulk quantities).

The information which follows has been assembled by Ph. Eur. staff from sources considered reliable, in particular from material provided in the ordinary way by the manufacturer or supplier. It has not been independently verified by the Ph. Eur. The accuracy of the information cannot therefore be guaranteed, nor does it constitute any expression of opinion by the Ph. Eur. 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 a Ph. Eur. CRS/BRP by professional persons having technical skill and at their own discretion and risk.

Any recipient of this Safety Data Sheet with responsibility for other persons in a workplace should determine the risks associated with the substance according to the conditions of use and should take appropriate measures, including provision of appropriate information to persons working with the substance.

Information given in sections 2, 3, 4, 5, 6, 9, 10, 11, 12 and 13 relates to the bulk substance and is not necessarily relevant to the CRS regarding the small quantities contained in vials. Information in sections 1, 7, 8 and 14 applies only to the CRS.

People working with reference material from biological origin (human or animal) should apply State-of-the-art precautions.
The 3\textsuperscript{rd} International Standard for Neomycin

1. The Standard

The 3\textsuperscript{rd} International Standard (IS) for Neomycin (ISA \_XXXX) consists of vials of freeze-dried neomycin. This preparation was established as the 3\textsuperscript{rd} IS for Neomycin by the Expert Committee on Biological Standardization of the World Health Organization in 2012.

2. Biological Activity

The standard was calibrated in an international collaborative study involving 10 laboratories from different countries, against the 2\textsuperscript{nd} IS for Neomycin.

\textbf{The assigned potency is 19050 IU per vial for the 3\textsuperscript{rd} IS for Neomycin.}

3. Use of the Standard

Dissolve the entire content of the vial with an exact 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$^\circ$C maximum during assays. Unused material must be discarded and not frozen for later use. Unopened vials should be stored at -20$^\circ$C.

The product in the vial is freeze-dried. Do not weigh out portions of the product, dissolve it preferably by injecting solvent through the rubber stopper while avoiding the generation of pressure within the vial which might lead to a loss of material when retracting the needle. The cake should dissolve rapidly. Care should be taken to avoid any loss and rinsing steps are recommended to ensure quantitative transfer into the volumetric flask.

4. Stability

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

5. References

Collaborative Study for the Establishment of the Third International Standard for Neomycin, WHO/BS/12.xxxx
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.