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**WHO Collaborative Study to Establish a Replacement WHO International
Standard for HCV RNA NAT Assays**

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Summary

In the collaborative study to establish the first International Standard for HCV RNA, there was no significant difference in potency between the two materials, AA and BB, which had been prepared from the same stock material but had been lyophilised in different runs. From the outset it was anticipated that sample BB would be considered as the candidate second International Standard and that a full collaborative study would not be considered essential as the candidate material had been fully characterised in the initial study to establish the first HCV International Standard.

Three laboratories using different NAT assays participated in a small collaborative study to assess the potency and the stability of the candidate replacement standard, sample BB in the original study, NIBSC code 96/798, compared with the current HCV International Standard (sample AA in the original study; NIBSC code 96/790). The current study investigated the relative stability of the two materials. There were no significant differences between the two materials stored at -20°C and no evidence of degradation over 5 years. Both materials also had similar profiles of degradation at higher temperatures. Based on this study, candidate material 96/798 is proposed as the second International Standard for HCV RNA NAT assays and assigned the same unitage as the first International Standard. The proposed second International Standard for HCV would therefore have a potency 50,000 IU/vial, which is equivalent to 10^5 IU/ml when a vial is reconstituted in 0.5ml.

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Introduction

The current International Standard for HCV RNA NAT assays, 96/790, was established in 1997 (WHO, 1999); attached as appendix 1; Saldanha *et al.*, 1999) and has been used widely to validate assays and to calibrate secondary standards and working reagents. The First International Standard consists of a batch of approximately 2300 vials, each containing lyophilised material equivalent to 0.5ml which was characterised in an international collaborative study together with two other materials; 96/798 (sample BB in the collaborative study), which was prepared from the same batch of material as the International Standard (sample AA in the collaborative study) but was lyophilised in a different run, and sample CC which was a liquid preparation. Sample AA was designated the first International Standard and assigned a potency of 50000IU/vial. In the collaborative study, there was no statistically significant difference between the potencies (titres) of samples AA and BB. As stocks of the current International Standard are now very low, sample BB, which has been held under identical conditions to the International Standard, has been assessed as a candidate replacement International Standard.

At the 12th SoGAT meeting (SoGAT 2000), the participants agreed that additional stability testing would be required before material 96/798 could replace the current International Standard as the 2nd International Standard, although a full collaborative study was not required. However, stability studies for NAT, both real time and accelerated degradation, have several limitations. For real time stability studies, changes in sensitivities of assays over time may be greater than degradation of samples thus masking any changes in degradation rates, while the required time scale for the study may be longer than the life of a high usage standard such as the HCV International Standard. For accelerated degradation studies, the high incubation temperatures may result in difficulty in reconstitution of samples. As reliable estimates require substantial degradation to occur, the time scale may be inadequate to observe this at lower temperatures. Sufficient time has now passed since the filling of the two materials (in 1996) and a new study can be done to confirm the equivalence of the present International Standard and material 96/798. Samples of both materials stored at +4°C and +20°C for over five years are available for studying the relative stability of the materials.

The objectives of the present study were to establish a replacement WHO International Standard for HCV RNA nucleic acid amplification (NAT) assays, to determine the stability of the candidate replacement material against the current HCV International Standard and to confirm the relative potency of the candidate replacement material against the current WHO International Standard, 96/790.

Materials and Methods

Preparation of materials

The first WHO International Standard for HCV RNA NAT assays, 96/790, and the proposed replacement material, 96/798, were prepared from the same starting material. Briefly, approximately 140ml of high titre HCV RNA and HCV antibody positive donation (genotype 1a) was thawed and added to 2040ml pooled cryosupernatant. The cryosupernatant was anti-HCV and anti-HIV negative. In addition, PCR assays on the cryosupernatant for HCV RNA, HAV RNA and parvovirus B19 DNA were negative. The diluted material was divided into two 1 litre aliquots and frozen in dry-ice. Each 1 litre aliquot was freeze-dried in a separate

run to give materials AA (the first WHO HCV International Standard, 96/790) and BB (96/798) by Q1 Biotech who are accredited for GLP.

For freeze-drying, one aliquot was thawed at 37°C with agitation until the sample had just thawed out. Thereafter, the sample was kept at 0°C. Approximately 2000 vials were filled with 0.5ml of sample and the cv of the fill volume for sample BB was 3.2%.

The vials were supplied by Adelphi Tubes (cat. no. VC002-13C) and were washed without detergent prior to sterilisation by oven baking at 180°C for 3 hours. Rubber seals (from Adelphi Tubes, cat. no. FD13) were immersed in 95% ethanol, 5% methanol for a minimum of 1 hour followed by autoclaving. The seals were placed on top of the filled vials, which were then loaded into the freeze-drier.

The shelves of the freeze-drier were pre-cooled to -40°C prior to loading the vials. The temperature was maintained at -40°C for at least 3 hours in the absence of any vacuum. After this initial period, a maximum vacuum was applied, whilst maintaining shelf temperature at -40°C, for a period of 90 hours. The condenser temperature was at or below -70°C. After this period, the temperature was raised from -40°C to +20°C over 20 hours while maintaining maximum vacuum. Once at +20°C, the freeze-drying chamber was back filled with N₂ and the vials sealed within the freeze-drier. A moisture trap was positioned between the N₂ and the freeze-drier to ensure dryness. N₂ gas with less than 5ppm O₂ was used. The vials were removed from the freeze-drier, crimp sealed with aluminum overseals and stored at -20°C.

Participants

Due to the limited supply of samples for this study, only three laboratories, code numbers 1 to 3, participated. These code numbers are not the same codes as used in the original collaborative study report.(Saldanha *et al*, 1997). The laboratories were chosen so that both commercial and in-house quantitative assays would be represented. In addition, the participating laboratories were able to complete the study in the given time frame. The list of participants and the types of assays used are shown in tables 1 and 2.

Organisation of the study

Participants were sent 4 panels, each containing 6 coded samples. These samples were ampoules of the current International Standard (NIBSC code 96/790) and the proposed replacement International Standard (NIBSC code 96/798, sample BB in the collaborative study to establish the first International Standard) stored at different temperatures. The samples had been stored at -20°C, +4°C and +20°C for a period of 5 years, 9 months and 18 days. Participants were requested to assay all samples concurrently, using a quantitative assay method, on 4 separate occasions. The composition of the panel is shown in table 3.

Data Received

All 3 laboratories provided results from 4 assays. Laboratories 1 and 3 used different in-house quantitative RT/PCR assays while laboratory 2 used the Roche quantitative Monitor version 2.0 assay. Laboratory 2 reported two replicate results for each assay, while laboratory 3 performed assays 2-4 concurrently. Laboratory 2 obtained results in IU/ml. Laboratory 1 obtained results in copies/ml, which were converted to and reported as IU/ml using a previously derived conversion factor. Laboratory 3 reported the results in 'in-house' units/ml. These were converted to IU/ml by taking potencies relative to the International Standard (-20°C samples). Therefore, for the final analysis, all results are expressed as IU/ml.

Statistical Methods

For each laboratory, overall mean estimates were obtained for each sample as the geometric mean (mean of \log_{10} estimates) of the individual assay estimates. The materials 96/790 and 96/798 were compared using paired t-tests independently for each temperature of storage, using the difference in estimates between the samples of 96/790 and 96/798 from each individual assay. The Arrhenius model of accelerated degradation (Kirkwood 1977; Kirkwood *et al* 1984) was applied.

Results

The laboratory mean estimates of \log_{10} IU/ml are shown in table 4. It can be seen that the samples stored at the higher temperatures are giving estimates that are lower than those from the samples stored at -20°C . Taking a mean across laboratories, there has been an average decrease of $\log_{10}0.9$ for the samples stored at $+4^{\circ}\text{C}$, and an average decrease of $\log_{10}1.9$ for the samples stored at $+20^{\circ}\text{C}$. However, the pattern is not consistent across laboratories, with laboratory 2 giving a lower estimate of the decrease across temperature of storage.

The estimates for the current IS (96/790) and the material BB (96/798) appear similar in all laboratories and at all temperatures. There is no significant difference between the IS and BB for samples stored at -20°C and $+4^{\circ}\text{C}$. There is a marginally significant difference (significant at 5% but not at 1%) between the IS and BB for the samples stored at $+20^{\circ}\text{C}$, with BB giving slightly higher estimates.

The data were analysed using the Arrhenius model for accelerated degradation. The Arrhenius model predicted a loss of 9% (or a $\log_{10}0.04$ decrease) per year for samples stored at -20°C , and 30% (or a $\log_{10}0.15$ decrease) per year for samples stored $+4^{\circ}\text{C}$, using either the pooled data or the data for the candidate replacement 96/798 alone. The data gave a statistically significant poor fit to the model however. In particular, the variability in results between laboratories for the samples stored at higher temperatures was not consistent with the simple degradation model. The observed loss at the higher temperatures may not therefore reflect the stability of samples stored at -20°C .

Discussion

The data from this study have shown a decrease in potency of the two materials (the current IS and the candidate replacement) after long term storage (5 years, 9 months and 18 days) at high temperature. However, there is no significant difference between the estimated potency of the materials 96/790 and 96/798 after long-term storage at -20°C or between the materials after long-term storage at $+4^{\circ}\text{C}$. There was a marginal difference between the samples stored at $+20^{\circ}\text{C}$ (significant at 5% but not 1%), with 96/798 exhibiting a slightly lower drop in potency.

The extent of the decrease in potency at higher temperatures was not consistent across laboratories, and the Arrhenius model for accelerated degradation did not give a good fit to the data. Although this model does estimate some limited degradation at -20°C , the estimates are questionable, and the observed decrease in potency at higher temperatures may not reflect the stability of samples stored at -20°C . The results from two laboratories both gave estimates above the assigned 5.0 \log_{10} IU/ml for the IS, which is not consistent with there being any significant degradation over the life of the standard.

The Instructions for Use of the First International standard indicate that if all of the reconstituted material is not used immediately, laboratories may aliquot the materials into suitable volumes which should be stored at -70°C .

Conclusions

In the original full collaborative study to establish the International Standard there was no significant difference between the two materials (Saldanha *et al*, 1999). The current study also found no significant difference between the two materials stored at -20°C , and a similar profile of degradation at higher temperatures. There is also no evidence of significant degradation at -20°C over the life of the current standard, from assays calibrated in IU/ml. As a result, we recommend that the material coded 96/798 should be adopted as the second International Standard, and assigned the same unitage as the first International Standard. The potency of the proposed second International Standard for HCV would therefore be 50,000 IU/vial which is equivalent to 10^5 IU/ml when a vial is reconstituted in 0.5ml

References

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Table 1. List of participants (and type of laboratory)

Manufacturer	Dr. C. Aberham/Dr. M. Gessner PC/Molecular Biology Baxter 20 Industriestrasse A-1220 Vienna Austria
Reference laboratory	Dr. J. Albrecht National Genetics Institute 2311 Pontius Avenue Los Angeles CA 90064 USA
EU Official Medicines Control Laboratory	Dr. G. Gentili/Dr. G. Pisani Laboratory of Immunology Istituto Superiore di Sanita Viale Regina Elena 229 00161 Rome Italy

Table 2. Protocols used by participants

Lab. code	Extraction method	RT/PCR	Primers	Detection
1	Ultracentrifugation +chaotropic agent with phenol/chloroform	In-house	Single (ND)	Chemiluminescent probes
2	Amplicor Monitor kit V 2.0	Amplicor Monitor kit V 2.0	Amplicor Monitor kit V 2.0	Amplicor Monitor kit V 2.0
3	QIAGEN UltraSens kit	In-house	Single (5'ncr)	Taqman technology

ND: not disclosed

5' ncr: 5' non-coding region

All assays used transcribed RNA calibrated against the International Standard for quantitation.

Table 3. Composition of panel for collaborative study

Sample number	Sample (temperature of storage)
1	IS, 96/790 (-20°C)
2	BB, 96/798 (-20°C)
3	IS, 96/790 (+4°C)
4	BB, 96/798 (+4°C)
5	IS, 96/790 (+20°C)
6	BB, 96/798 (+20°C)

Table 4. Laboratory mean estimates (\log_{10} IU/ml)

Temperature of incubation	Laboratory 1		Laboratory 2		Laboratory 3	
	IS (96/790)	BB (96/798)	IS (96/790)	BB (96/798)	IS (96/790)	BB (96/798)
-20°C	5.07	5.06	5.27	5.15	5.00	5.05
+4°C	3.93	3.99	4.50	4.63	4.12	3.98
+20°C	2.63	2.85	3.44	3.78	3.14	3.31

Virus Reference) and assigned a value of 1.1% 472-C nucleotide to the contents of each vial.

The Committee encouraged the development of similar reference materials for type 1 and 2 poliovirus (Sabin).

Hepatitis C virus RNA

The Committee noted a proposal to establish a reference material for hepatitis C virus RNA (BS/97.1861). The proposal was based on the results of a collaborative study performed by 22 laboratories in 11 countries. It further noted data showing that the candidate material had adequate stability in accelerated stability studies and was informed that real-time stability studies would also be carried out.

The Committee established the preparation, coded AA, as the International Standard for Hepatitis C Virus RNA, and assigned a potency of 50000 International Units to each vial. It requested that the additional stability data supplied by the study coordinators be appended to document BS/97.1861.

The Committee further noted the high variability of the fill volume (coefficient of variation = 8.39%), which did not meet the strict specification stipulated in the Guidelines for the preparation, characterization and establishment of international and other standards and reference reagents for biological substances (WHO Technical Report Series, No. 800, 1990, Annex 4). As the material was highly infective, it was not possible to process it in the usual facilities at the National Institute for Biological Standards and Control, Potters Bar. As a result of the inherent variability of the gene amplification technology, assays on ampoules with the highest and lowest fill volumes did not, however, show differences.

The Committee also noted the availability of another material, coded BB, as a possible future replacement of AA, and recommended that it should also be tested in the ongoing stability studies.

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