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**A Collaborative Study to value assign the WHO 2nd International
Standard for High Molecular Weight Urokinase (11/184)**

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This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by 01 October 2012 and should be submitted electronically to the Responsible Officer: Dr Ana Padilla at email: padillaa@who.int, with a copy to Dr David Wood at email: woodd@who.int.

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Summary

The existing WHO 1st International Standard (IS) for High Molecular Weight Urokinase, 87/594, is running out and a replacement is required. A preparation of high molecular weight urokinase was provided by a manufacturer, formulated, filled and freeze dried into 4700 sealed glass ampoules coded 11/184. An international collaborative study was organised to calibrate the candidate 2nd WHO IS for High Molecular Weight Urokinase, 11/184 against the current IS, 87/594, which has an assigned potency of 4300 IU per ampoule. A total of 15 laboratories were recruited to take part in the study and 14 laboratories from 10 different countries returned results comprising 55 independent assays. A laboratory geometric mean was calculated from each laboratory's results and an overall geometric mean of laboratory means was used to assign a potency to the candidate replacement IS. In this way, the candidate IS 11/184 was determined to have a potency of 3238 IU/ampoule with an overall geometric coefficient of variation (GCV) of 7.1 %.

Proposal: Preparation 11/184 is proposed as the WHO 2nd International Standard for High Molecular Weight Urokinase with a potency of 3200 IU per ampoule.

Introduction

Urokinase is a serine protease, plasminogen activator that has been used clinically as a thrombolytic agent. Physiologically, urokinase is produced in a zymogen form, single chain urokinase or pro-urokinase, that is cleaved by plasmin to produce the active form of urokinase, also known as two chain urokinase type plasminogen activator or uPA. However, further degradation products are generated, which may have different activities and the major forms of two chain urokinase are designated high molecular weight urokinase (Mwt 54 kDa) and low molecular weight urokinase (Mwt 33 kDa) [1]. There have been 2 WHO International Reference Preparations used to assign potency to urokinase products. The first preparation produced at NIBSC in the 1960s was a mixture of high and low molecular weight urokinase forms, and was coded 66/34. This was replaced in 1989 by the WHO 1st International Standard (IS) for High Molecular Weight Urokinase, 87/594 a more homogeneous preparation of high molecular weight urokinase developed to reflect the improved quality of urokinase products [2]. This IS is now becoming depleted and a replacement is needed. This report describes the preparation of a candidate replacement IS and the subsequent international collaborative study that was organised to calibrate the replacement relative to the existing WHO IS, 87/594. As was the case with the previous reference preparations, the candidate IS is calibrated in International Units (IU) which are determined in fibrinolytic assays. Furthermore, pharmacopoeia assays for urokinase therapeutic products (e.g. the European Pharmacopoeia) specify fibrinolytic assays in some form for potency determinations. In order to provide additional information to end users of the IS and in line with progress towards dual labelling of fibrinolysis standards in IU and molar concentrations where possible, active site titration assays were performed on the bulk material and estimates for active site concentration of urokinase will be provided for the filled ampoules. The value for concentration of active sites is not intended for endorsement by WHO, but will be included in the Instructions for Use documentation, for information only. The project to replace the existing WHO IS, 87/594, has been endorsed by the Scientific Standardisation Committee (SSC) of the International Society for Thrombosis and Hemostasis (ISTH) and is run in conjunction with the Fibrinolysis Subcommittee. Materials, design of the study and participants in the study are detailed below. The project for replacement of the 1st IS for High Molecular Weight Urokinase was endorsed by the ECBS in October 2010.

Materials

One manufacturer kindly donated a sample of a batch of therapeutic urokinase product, as a frozen solution of urokinase purified from human urine, to be used in the preparation of the candidate WHO 2nd IS for High Molecular Weight Urokinase. According to the certificate of analysis provided by the manufacturer the product was tested and found negative for anti hepatitis B and C antibodies and antibodies to HIV 1 and 2. The solution was approximately 11 mg/ml urokinase with a specific activity >100 000 IU/mg protein and HPLC and SDS PAGE analysis showed the urokinase present to be >95% high molecular weight two chain form.

On receipt, the frozen solution was stored at -80 °C until required and was thawed and formulated for filling into ampoules. The bulk solution prepared for the definitive fill of the candidate replacement IS was composed of 5 L of 10 mM Hepes buffer pH 7.4, containing 0.15 M NaCl, 5 mg/ml human albumin and 30 µg/ml urokinase. The solution was thoroughly mixed and maintained at 4 °C during dispensing in 1 ml aliquots into glass ampoules before freeze drying and sealing. All processing steps were performed at, and storage of ampoules at -20 °C, will be at, NIBSC, S. Mimmis, Herts, UK. The end result was a batch of ampoules of candidate IS coded 11/184. The characteristics of this batch are provided in Table 1. The uncertainty of the content of the ampoules is given by the variance of the fill as shown in Table 1.

Design of the study

The study was simple in design, including only one Standard, the current WHO 1st IS for High Molecular Weight Urokinase, 87/594 and one Test sample, the candidate WHO 2nd IS for High Molecular Weight Urokinase, 11/184. Participants were requested to perform 4 independent clot lysis or other suitable fibrinolytic assays to determine the potency of the Test sample relative to the Standard. Five ampoules of both Standard and Test preparations were provided, sufficient for 4 independent assays and an optional 5th assay (or a repeat in case of error). Guidelines were provided on randomisation and other key assay features, such as the inclusion of sufficient replication to permit statistical testing for deviations of dose response curves from linearity and parallelism. It was suggested that a four point dose response curve with doubling dilutions was optimal, though not essential. Participants were able to use their normal routine assays, but an alternative clot lysis assay method was provided which could be used with a few common reagents. This method also included general advice for optimising randomisation of samples, dilution ranges and other practical guidance. A copy of the study protocol and assay method is included in Appendix 1.

Participants were requested to return raw data for analysis to NIBSC, which could be in the form of times to clot lysis, or raw clot lysis curves as microtitre plate readouts or Excel spreadsheets, so that clot lysis times could be calculated and processed from the raw data. Participants were invited to complete their own analysis if desired for comparison with NIBSC analysis, but their analysis would be for information only.

In line with normal practice, raw data from each assay were used to calculate the relative potency of Test 11/184 against the Standard 87/594, by parallel-line analysis using the CombiStats software [3]. Deviations from linearity and parallelism were considered significant at the 5% level ($p < 0.05$). Data from all valid assays were combined to generate an unweighted geometric mean for each laboratory and these laboratory means were then used to calculate an overall unweighted geometric mean potency for 11/184. Variability between assays and laboratories has been expressed using geometric coefficients of variation ($GCV = \{10^s - 1\} \times 100\%$ where s is the

standard deviation of the log transformed potencies). Grubbs' Test was applied to the log transformed laboratory mean potencies in order to detect any significant outliers. Comparisons between methods have been made by unpaired t-tests of log transformed laboratory mean potencies.

Participants

A total of 15 laboratories agreed to take part in the study and 14 laboratories returned data. Participants included 9 academic, 3 manufacturing and 2 regulatory laboratories from 11 different countries (3 UK, 2 USA, France, Netherlands, Belgium, Italy, Hungary, India, China, Japan, Australia). All laboratories were assigned a code number and the names and addresses of participants are listed in Appendix 2, Acknowledgements, where the order of listing is not the same as the laboratory code number.

Results and Discussion

Assay methods

Participants were requested to provide details of their methods when returning results (see Appendix 1 for questionnaire). All methods used were some form of clot lysis assays, mostly in a microtitre plate format (12 out of 14 laboratories returning results) where fibrin turbidity was monitored during clotting and lysis. From these curves a time to 50% clot lysis was determined usually by calculating the time from the first reading (the clotting phase) to the time where absorbance was half way between the absorbance peak (end of clotting phase) and the final absorbance in the well (complete lysis). Alternative measures of 50 % lysis as the time from peak absorbance to the time to 50% drop in absorbance gave no improvements in the data analysis and in fact generally produced more scattered results. One laboratory reported time to 50% lysis as the time between 50% clotting and 50% lysis, as is their usual measure. The remaining laboratories not using microtitre plate methods (2/14) formed fibrin clots in tubes and measured the time to lysis by following some physical property to show the collapse of the clot.

Of the 14 laboratories returning results in the study, 13 used human fibrinogen in some form and 1 laboratory used bovine fibrinogen. The majority of laboratories used a purified system to generate clots and follow clot lysis, mixing purified fibrinogen, plasminogen, thrombin with the urokinase dose range (10 out of 14 laboratories). Reagents were purchased from various commercial sources or home made. In this group using purified reagents, eight of the 10 laboratories followed the optional method provided to participants in most of the detail, though there were some deviations by some laboratories from the recommendations for randomisation of sample preparation and plate layout between assays. Two laboratories also noted slow clotting and lysis profiles and increased the concentrations of urokinase in the reaction mixtures of Standard and Test preparations to achieve complete lysis within a reasonable time (under 5 hours for the lowest dose of urokinase was recommended).

The 4 laboratories that did not use purified fibrinogen used human plasma to provide fibrinogen, plasminogen and thrombin though there were differences between these groups in the ways clotting was initiated in the anticoagulated plasma.

Statistical Analysis, Exceptions and Deviations

Fourteen laboratories returned data from a total of 57 independent assays and data from 55 assays were used after two laboratories, numbers 4 and 14, withdrew 1 assay each. Most laboratories reported no difficulties in performing the assays and all analysis was performed using log transformed lysis time versus log urokinase concentration. The following exceptions and deviations are reported.

Several sets of data from different laboratories were highlighted by the analysis software as having significant non-linearity ($p < 0.05$). However, visual inspection invariably suggested that this significance was an anomaly due to tight replication (and therefore underestimation of the residual error), sometimes a problem encountered with precise assay methods. In such cases, non-parallelism was assessed using deviations from linearity as an alternative residual error. No laboratory results were rejected for deviations from linearity or parallelism after this procedure was followed.

A dilution range of four doses made by 2-fold serial dilutions (an 8 fold range overall) was suggested in the optional method included with the study protocol. Laboratories 1, 8 and 15 used their routine in-house methods which applied a narrower range of dilutions for Standard and Test samples, 1.5 –fold, 1.4-fold, and 2.4-fold, overall, respectively. Laboratories 1 and 8 included four doses and laboratory 15 three doses of urokinase. A narrow range of dilutions may have contributed to assay variability, and could mask deviations from linearity and parallelism. However, no deviations from linearity and parallelism were observed and results from all four assays performed by these laboratories were included in the data analysis.

Laboratory 4 submitted 5 sets of data, however, assay 3 was significantly non-linear with a limited dose response for the highest concentrations of urokinase in both Sample and Test wells. On further discussion with the laboratory they were concerned that sample dilutions may have been mixed up on this plate and requested the assay be withdrawn.

Laboratories 2 and 3 observed long clot lysis times in both Standard and Test samples meaning that the lowest concentrations of urokinase did not reach 50% lysis within 5 hours, as recommended. As a consequence both laboratories increased the concentration of urokinase in their dilution range from that suggested in the method provided. All assays from laboratories 2 and 3 were valid and included in the analysis. Laboratory 14 also observed slow reactions but retained the suggested dilution regimes, which meant that the lowest concentrations of urokinase did not achieve 50% lysis. Consequently data were analysed using a three point dose response curve, for the three highest concentrations for both Standard and Test samples in all assays. Laboratory 14 was also concerned about the arrangement of Standard and Test samples on the microtitre plate in their second assay and withdrew this assay, thus only three assays were analysed from laboratory 14.

Laboratory 9 performed the method provided but results from both Standard and Test samples in all assays were non-linear due to a poor dose response at the lowest concentrations of urokinase (longest lysis times). This was not due to plate effects as the arrangement of samples was randomised as suggested in the protocol and all further investigations failed to identify the source of this anomaly. Otherwise all assays from laboratory 9 were without problems and were analysed using a 3 point dose response curve, for the 3 highest concentrations for both Standard and Test samples which gave acceptable linearity and parallelism.

Clotting and lysis curves from laboratory 10 had a sparse distribution of few data points making it difficult to identify precisely the time point for 50% clot lysis. To overcome this problem, curves were fitted to the data provided and time to 50% lysis determined from the fitted curves. Data from all four assays returned were included in the final analysis.

Summary of Results

A summary of results is presented in Table 2. The overall potency of 11/184, the candidate WHO 2nd IS was 3238 IU/ampoule, with an inter-laboratory GCV of 7.1% (n=14). In general, individual laboratory results and intra-laboratory variation was good, only two laboratories reported GCV values >10 % and half of laboratories had GCV values <5%. Furthermore, 10 of the 14 laboratories determined a potency for 11/184 that was within 5% of the overall mean value.

If results are broken down by methods it can be calculated that laboratories using purified assay components (10 laboratories using purified human or bovine fibrinogen) obtained a potency of 3223 IU/ampoule (GCV 6.2%) and laboratories using human plasma (four laboratories) obtained a potency of 3277 IU/ampoule (GCV=10.0%). The difference in potency between these groups was not significant (p>0.05), however, it should be noted only four laboratories used plasma-based methods. Those laboratories that followed the optional method provided (eight laboratories) obtained a potency of 3169 IU/ampoule with a low GCV of 3.8%, suggesting this is a robust method, even when being performed in laboratories that do not use it routinely.

Laboratory 15 was the only one to use bovine fibrinogen rather than human fibrinogen (either purified or from plasma) and reported the highest potency, 15% from the overall mean value. However, this result was not found to be a significant outlier. Furthermore, additional assays at NIBSC using the precise method provided in the study but substituting bovine fibrinogen for human fibrinogen produced a potency that was 3.3% lower and not significantly different from the potency derived for 11/185 versus 87/594 with human fibrinogen. Thus there is no evidence to suggest that bovine fibrinogen might be responsible for the higher potency reported by laboratory 15.

The overall potency calculated for 11/184 using the data provided is 3238 IU per ampoule which is 75.3% of the potency of 87/594 (4300 IU per ampoule). This lower potency of the candidate replacement IS will not cause any problems for assay methods, since most assay methods require a much lower urokinase concentration range (the optional method provided for the study used a range of urokinase of 8.6, 4.3, 2.15 and 1.08 IU/ml in the reaction mixtures, for example). The difference in activity between preparations 87/594 and 11/184 may be due to differences in protein supplied (in both cases only the data provided by the manufacturer was used to guide the amount used in the formulation) with the possibility of small additional losses during processing and manufacture of the new IS.

Study results are also presented in graphical form as a histogram of laboratory means, colour coded by method, in Figure 1.

Stability Studies

Long term stability of Candidate WHO 2nd International Standard 11/184

In line with usual practice, ampoules of 11/184 were subjected to storage at elevated temperatures (4, 20, 37, 45 and 56 °C) for prolonged periods of time to investigate long term

stability in comparison with ampoules stored at -20 °C. From these studies and application of the Arrhenius model, predictions will be made for the long term stability of 11/184 -20 °C and for shorter periods at ambient temperatures as experienced during shipping [5]. These studies will be carried out over the coming years but assays have been performed on the first time point for ampoules stored at elevated temperatures (20, 37, 45 and 56 °C for 6 months). Assays were performed in one laboratory (NIBSC) using the method provided with the study (see appendix 1). A series of 5 assays were performed using 2 sets of ampoules and solutions of reconstituted 11/184 were kept on ice between assays. A summary of data collected for each individual assay and for the potencies calculated from the combined data for all assays is shown in Table 3

All results indicate that the urokinase activity in ampoules of 11/184 is very stable during storage at elevated temperature and no measureable degradation can be found after 6 months at temperatures up to 45 °C. After 6 months at 56 °C there is a loss of activity of around 16 %. The loss of activity at a single temperature at this one time point does not allow a prediction of stability at minus 20 °C to be made and more time is required to allow for further loss of activity at more temperatures. These studies will be continued.

Bench stability

Bench stability of solutions after reconstitution of the contents of ampoules of 11/184 was investigated over periods of several hours as might be experienced during use in routine assays. Table 4 shows a summary of results from fibrinolysis assays on solutions from ampoules after reconstitution and analysis immediately (within 1 hour) and after 24 hours and 4 days at 4 °C. Also shown are results using ampoules filled with an alternative formulation that contained no albumin or other protein besides urokinase, but instead used sucrose and mannitol as bulking agents. This protein-free formulation was proposed and investigated in trial fills as an alternative to filling with albumin as this would allow for further physicochemical studies on 11/184. It is clear from the results shown in Table 4 that in the albumin formulation used in 11/184, urokinase activity is very stable after reconstitution. Even after 24 hours at 4 °C there is only a few percent loss of activity (not statistically significantly different from a freshly reconstituted ampoule within the precision of the method), and even after 4 days at 4°C only 21.6 % of activity was lost. However, if filling and freeze drying was performed without albumin in the formulation, the results shown in Table 4 suggest the product is unstable.

Molar Concentration of Urokinase

End users of protease International Standards calibrated in IU often request information on the molar concentration of active enzyme in the preparation. This value is not generally determined for IS however molar concentrations of protease enzymes can be measured by active-site titration, provided a suitable titration substrate is available. This approach was used successfully to calibrate the WHO 1st IS for alpha-1-antitrypsin (AAT) by first determining the active enzyme concentration in a batch of trypsin, using a standard method with the colourimetric active site titration substrate 4-Nitrophenyl 4-guanidinobenzoate hydrochloride (NPGb) [6]. For the present study on urokinase active-site titration assays were performed at NIBSC with stock solutions of the candidate urokinase preparation against a fluorimetric active site titration substrate, 4-Methylumbelliferyl 4-guanidinobenzoate hydrochloride hydrate (MUGb). MUGb has the advantage of being more sensitive than NPGb and therefore requires lower enzyme concentrations. A stopped flow unit was used to achieve rapid mixing of urokinase with MUGb, and this was coupled to a fluorimetric spectrometer to simultaneously provide an output trigger to start kinetic measurements to capture the required burst of product release from MUGb mixed

with urokinase. Three dilutions of the urokinase candidate starting material were made within the concentration range 500 - 1500 nM, which is appropriate for MUGB titrations. Active-site titrations were performed at each of the urokinase dilutions including several replicates, and the magnitude of the burst was calculated relative to a standard curve for 4-methylumbelliferone (the fluorophore product released from MUGB substrate). The molar concentration of the starting material was calculated to be 139 μM (GCV=4.4 %; n=14) after taking the geometric mean of all titrations corrected for the initial dilution factors. Based on this value we estimate the concentration of active urokinase in the final ampouled material to be 380 nM after reconstitution, equivalent to approximately 21 μg per ampoule. This estimate is in line with the expected specific activity and the estimate for the previous IS, 87/594, in the range of 140-150 IU/ μg . The molar concentration of urokinase will not be submitted for endorsement by WHO for labelling of the candidate WHO 2nd IS for High Molecular Weight Urokinase, 11/184, which will be in IU only. However, it is proposed that this information on molar concentration will be included in the Instructions for Use that accompanies the IS and this may be helpful for certain applications.

Conclusions

The existing WHO 1st IS for High Molecular Weight Urokinase 87/594 has an assigned potency of 4300 IU/ampoule. A replacement IS was prepared, coded 11/184, and calibrated against the existing IS. The calibration exercise was well performed and relatively trouble-free resulting in a potency assignment of 3238 IU/ampoule for the 11/184. The overall inter-laboratory GCV of 7.1 % was low indicating a good level of agreement of results between participating laboratories. No significant differences were observed between methods, suggesting the replacement Standard is suitable for methods using purified components or plasma.

Proposal

Preparation 11/184 is proposed as the WHO 2nd International Standard for High Molecular Weight Urokinase with a potency of 3200 IU per ampoule.

Participants' and Experts' comments on the report are presented below in Appendix 3 and 4.

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Tables**Table 1. Characteristics of the filled batch of ampoules of candidate IS 11/184.**

Parameter	Result
Ampoules available	4734
Mean mass of fill (CV) [no. tested]	1.0099 g (0.14 %) [173]
Mean dry weight (CV) [no. tested]	0.017 g (3.39 %) [6]
Mean residual moisture (CV) [no. tested]	0.40 % (26.71 %) [12]
Mean oxygen head space (CV) [no. tested]	0.23 % (61.19 %) [12]

Table 2 summary of data analysis for potency determination of candidate WHO 2nd IS for High Molecular Weight Urokinase 11/184 against the WHO 1st IS, 87/594.

Laboratory number	Assays	Potency of 11/184		Intra-laboratory		Method	% difference from overall mean
		IU/ampoule		GCV %			
1	4	3567		15.4		HP	10.2
2	4	3085		5.3		<i>HF</i>	-4.7
3	4	3069		6.8		<i>HF</i>	-5.2
4	4	3072		8.7		<i>HF</i>	-5.1
5	4	3199		2.9		HF	-1.2
6	4	3156		0.8		HP	-2.5
7	4	3108		8.4		<i>HF</i>	-4.0
8	4	3514		4.2		HP	8.5
9	4	3338		3.0		<i>HF</i>	3.1
10	4	2915		37.2		HP	-10.0
11	4	3196		0.4		<i>HF</i>	-1.3
12							
13	4	3139		5.5		<i>HF</i>	-3.1
14	3	3350		4.2		<i>HF</i>	3.5
15	4	3724		2.0		BF	15.0
Overall (n=14)		Geometric mean	3238 IU/ampoule	Inter-lab GCV%	7.1		

Lab 12 results not returned due to difficulty in obtaining reagents

Method code: HF=purified human fibrinogen (italics indicate followed optional protocol method provided), HP= human plasma assay system, BF=bovine fibrinogen assay system.

Table 3. Potency determinations for 11/184, candidate WHO 2nd IS for High Molecular Weight Urokinase after 6 months at elevated temperature. All potencies were measured against ampoules stored at -20 °C, assigned a potency of 100% (3200 IU/ml)

Assay number	% Activity remaining (95% confidence interval)			
	20 °C	37 °C	45 °C	56 °C
1	98.3 (87.5-110.4)	102.5 (91.2-115.2)	100.3 (89.3-112.7)	84.7 (75.4-95.2)
2	100.4 (94.1-106.8)	103.3 (97.0-110.1)	100.3 (94.2-106.8)	82.4 (77.3-87.7)
3	104.7 (92.5-118.6)	103.9 (91.7-117.6)	101.1 (89.3-114.5)	89.8 (79.3-101.7)
4	101.0 (89.5-113.8)	100.1 (88.7-112.8)	110.2 (97.8-124.3)	91.8 (81.4-103.5)
5	97.5 (87.7-109.5)	101.5 (91.2-113.0)	94.3 (84.7-104.9)	72.8 (65.4-81.1)
Combination of potencies	100.3 (96.9-103.9)	102.3 (100.4-104.1)	101.1 (94.3-108.4)	84.1 (75.1-94.1)

Table 4 Stability of urokinase activity in ampoules of 11/184 after reconstitution and storage in the cold and of an alternative formulation without albumin. Potencies were determined against the WHO 1st IS for High Molecular Weight Urokinase, 87/594 and are also expressed as a percentage of the activity of a freshly reconstituted ampoule of 11/184, using the consensus potency of 3200 IU/ml (100%).

Conditions	% Remaining activity IU/ml (% of 11/184)	
	11/184 (Albumin formulation ¹)	SS-324 (Mannitol/sucrose formulation ²)
Fresh	3200 (100%)	1898 (59.3%)
8 hours on ice	3098 (96.8%) ³	N/A
24 hours at 4°C	3060 (95.6%) ³	1019 (31.9%)
4 days at 4°C	2510 (78.5%)	780 (24.4%)

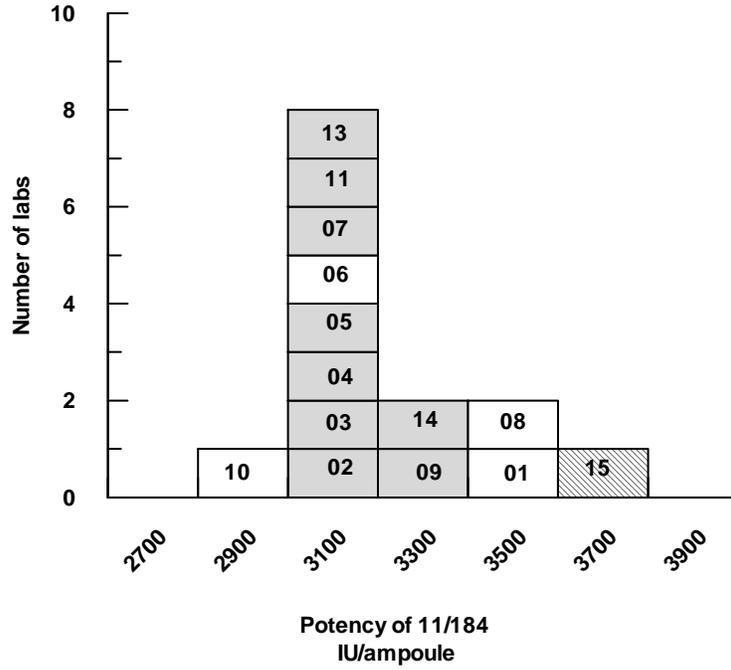
¹Formulation was 10 mM HEPES, pH 7.4 containing 0.15M NaCl and 5 mg/ml human albumin

²Formulation was 10 mM HEPES, pH 7.4 containing 40 mg/ml mannitol and 10 mg/ml sucrose.

³Overlapping 95% confidence interval with freshly reconstituted 11/184

Figures

Figure 1. Histogram summarising the distribution of potency determinations by laboratory and method for the calibration of candidate WHO 2nd IS for High Molecular Weight Urokinase, 11/184 against the current WHO 1st IS, 87/594. (shaded squares are assays with human fibrinogen, hatched square is bovine fibrinogen and open squares are human plasma).



Appendix 1 Study protocol

Example fibrin microtitre plate clot lysis assay:

Urokinase 87/594 standard versus urokinase 11/184 test

Background

Fibrin clots are prepared in a microtitre plate by mixing solutions as follows

60 µl Fibrinogen + Plasminogen	40 µl Thrombin + Urokinase
Mix	
100 µl clot formation and lysis	

Clotting and lysis are monitored in the plate reader and data may be analysed in a number of ways, often the time to 50% lysis of the clot is used as the endpoint.

In this assay we include 4 doses of urokinase made up using doubling dilutions. All other reactants remain constant.

The conditions of this assay have been arranged so clot lysis is complete within around 2 hours. Significantly longer times should be avoided to avoid problems caused by clots drying out during the assay. If reactions are significantly slower (or faster) it may be necessary to adjust the urokinase concentration range.

Method using fibrinogen solution.

Reagents

Buffers (buffers are from the Streptokinase solution assay, EP method)

A 0.5 M Tris from tables. 0.5 M Tris (30.29 g, 121.14 g/mol) + 183 ml of 1 M HCl made up to 500 ml (pH is 7.7 at RT). Can be stored at 4 °C.

B Made from buffer A. Is 10 mM Tris HCl with 100 mM NaCl and 0.01% Tween 20 (5 ml of buffer A + 1.46 g NaCl and 250 µl of 10% Tween 20 up to 250 ml). Can be stored at 4 °C

C Buffer B + HSA 1 mg/ml (500 µl of 20% albumin in 100 ml). Made fresh each day.

D Buffer C + thrombin (buffer C + 5 µl per ml of thrombin of 100 IU/ml, or ~ 1 µM). Made fresh each day.

Fibrinogen

One bottle of 1 g fibrinogen is dissolved in 20 ml of buffer B at RT. The contents are stirred gently for 30 mins then dispensed as 0.5 ml aliquots in eppendorfs and flash frozen before storage at -40 °C. This solution is 50 mg/ml fibrinogen. Do not refreeze thawed aliquots.

Glu-Plasminogen

From Hyphen (for example), made up to 1 mg/ml in water.

Thrombin

Use thrombin at ~100 IU/ml (around 1 µM) and frozen aliquots are acceptable. Bovine thrombin (eg Thame Diagnostics) may also be used, or some other good quality thrombin.

Plasminogen activator

Urokinase 87/594, 4300 IU/ml (30 µg/ampoule) and 11/184 Urokinase of a similar concentration.

Method

Activator + Thrombin Solution

The Standard, 87/594 and Test, 11/184 are of similar potency and may be treated in the same way. Each assay uses 1 ampoule of 87/594 and 1 ampoule of 11/184.

Plasminogen activators in ampoules are reconstituted in water and are diluted in buffer D.

Make up a series of dilutions of the activator. For example:

Ampoules dissolved in 1 ml water	1st dilution in buffer D	2nd dilution in buffer D
87/594	50 µl made up to 1 ml J	50 µl ml made up to 0.5 ml K
11/184	50 µl made up to 1 ml P	50 µl ml made up to 0.5 ml Q

Aliquots of 200 µl of **K** and **Q** are dispensed as shown in the plate below and used to make a series of dilutions, mixing 100 µl urokinase solution with 100 µl buffer D, down the plate to produce the dilution series **K L, M and N** for **87/594**

Similarly, a series of 4 doses are made from **Q** by mixing equal volumes of urokinase solution and buffer D to produce solutions **Q R, S and T** for **11/184**

A replicate set of dilutions is made from the same ampoule of 87/594 and 11/184 to generate solutions **K', L', M' and N'**; and **Q', R', S' and T'**.

The serial dilutions may be made up in a microtitre plate as follows (a multichannel pipette is useful) and incubated at 37 °C:

	Neat	K	Q	K	Q	K'	Q'	K'	Q'		
	1:2	L	R	L	R	L'	R'	L'	R'		
	1:4	M	S	M	S	M'	S'	M'	S'		
	1:8	N	T	N	T	N'	T'	N'	T'		

Fibrinogen + Plasminogen Solution

A 0.5 ml aliquot of Calbiochem fibrinogen solution (50 mg/ml) is mixed with 2.0 ml of buffer B and warmed to 37°C. Immediately before clotting is to begin, 40 µl of glu-plasminogen, 1 mg/ml, is added.

Clotting and Fibrinolysis

To begin the clotting reaction, 40 µl of urokinase +thrombin solutions, K-L, Q-T, K'L' and Q'-T' are transferred to the lower half of the plate as shown below to give solutions k-l, q-t, k'-l' and q'-t' (rows E-H, columns 3-10)

Then 60 µl of the fibrinogen + plasminogen solution is dispensed into the same wells (rows E-H, columns 2-10). These solutions and the plate should be pre-warmed to 37 °C.

		K	Q	K	Q	K'	Q'	K'	Q'	Rows A-D Dilutions of urokinase – thrombin solutions
		L	R	L	R	L'	R'	L'	R'	
		M	S	M	S	M'	S'	M'	S'	
		N	T	N	T	N'	T'	N'	T'	
		k	q	k	q	k'	q'	k'	q'	Rows E-H Mix urokinase/thrombin with fibrinogen/plasminogen to form clots and monitor lysis
		l	r	l	r	l'	r'	l'	r'	
		m	s	m	s	m'	s'	m'	s'	
		n	t	n	t	n'	t'	n'	t'	

The plate is transferred to a plate reader as quickly as possible and absorbance read and recorded over time at 405 nm at 30 s intervals at 37 °C for up to 5 hours or until the clots are completely lysed.

General principles

Data Reporting

To calculate the potency of the Test sample, 11/184, relative to the Standard 87/594, raw data are needed. Acceptable data forms are:

Time to 50% lysis of clots in all wells (including all replicates, and not just the means)

Raw data in the form of clot lysis profiles (a column of time versus columns of absorbance data) for all clot lysis curves as an Excel spreadsheet or a Softmax Data File (.pda extension) will be acceptable.

You may calculate your own potency values for the Test Sample relative to the Standard if you wish but do please send us the data you use.

Plate Layout

We request you perform a series of 4 independent assays each one using a fresh ampoule of 87/594 and 11/184. To avoid systematic errors due to dilution errors or plate effects you should vary the arrangement of samples on the plate. A suitable arrangement over the 4 assays is shown as follows.

Red = Standard, 87/594

Blue = Test, 11/184

prime letters indicate a second independent set of dilutions from the same ampoule

Rows A-D (cols 3-10) are used to make the final set of dilutions

Rows E-H (cols 3-10) are used to mix reactants and record measurements

Assay

1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E			1/1	1/1	1/1	1/1	1/1'	1/1'	1/1'	1/1'		
F			1/2	1/2	1/2	1/2	1/2'	1/2'	1/2'	1/2'		
G			1/4	1/4	1/4	1/4	1/4'	1/4'	1/4'	1/4'		
H			1/8	1/8	1/8	1/8	1/8'	1/8'	1/8'	1/8'		

Assay

2

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E			1/1	1/1	1/1	1/1	1/1'	1/1'	1/1'	1/1'		
F			1/2	1/2	1/2	1/2	1/2'	1/2'	1/2'	1/2'		
G			1/4	1/4	1/4	1/4	1/4'	1/4'	1/4'	1/4'		
H			1/8	1/8	1/8	1/8	1/8'	1/8'	1/8'	1/8'		

Assay

3

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E			1/8	1/8	1/8	1/8	1/8'	1/8'	1/8'	1/8'		
F			1/4	1/4	1/4	1/4	1/4'	1/4'	1/4'	1/4'		
G			1/2	1/2	1/2	1/2	1/2'	1/2'	1/2'	1/2'		
H			1/1	1/1	1/1	1/1	1/1'	1/1'	1/1'	1/1'		

Assay

4

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E			1/8	1/8	1/8	1/8	1/8'	1/8'	1/8'	1/8'		
F			1/4	1/4	1/4	1/4	1/4'	1/4'	1/4'	1/4'		
G			1/2	1/2	1/2	1/2	1/2'	1/2'	1/2'	1/2'		
H			1/1	1/1	1/1	1/1	1/1'	1/1'	1/1'	1/1'		

Data Analysis

Data analysis will be performed at NIBSC. The most likely endpoint we will use will be time to 50% clot lysis. Results for clot lysis time versus dose of urokinase for Standard and Test will be prepared for Combistats analysis. The potency and 95% confidence intervals for the Test, 11/184 relative to the Standard 87/594, will be calculated along with estimations of the likelihood of deviations from linearity and parallelism.

Example Assay Result.

Times to 50% lysis (seconds)

Assay 1

	K	Q	K	Q	K'	Q'	K'	Q'	Dilutions of urokinase – thrombin solutions
	L	R	L	R	L'	R'	L'	R'	
	M	S	M	S	M'	S'	M'	S'	
	N	T	N	T	N'	T'	N'	T'	
	1620	1800	1710	1710	1590	1680	1530	1650	Mix reactants to form clots and monitor lysis
	2340	2760	2490	2790	2580	2820	2130	2430	
	3450	4350	3780	4140	3870	4110	3660	3870	
	6420	6510	5580	6630	6360	6600	5940	6150	

Standard				
Id.	87/594 in 1 ml water			
Ass. pot.	4300 iu/ml			
Pre-dil. 1	0.05ml/1.0ml			
Pre-dil. 2	0.05ml/0.5ml			
Doses	(1)	(2)	(3)	(4)
1/1	1620	1710	1590	1530
1/2	2340	2490	2580	2130
1/4	3450	3780	3870	3660
1/8	6420	5580	6360	5940

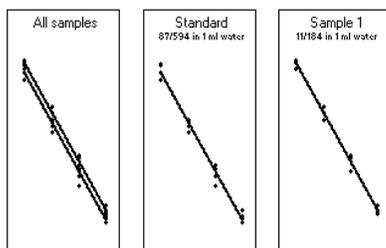
Sample 1				
Id.	11/184 in 1 ml water			
Ass. pot.	4300 iu/ml			
Pre-dil. 1	0.05ml/1.0ml			
Pre-dil. 2	0.05ml/0.5ml			
Doses	(1)	(2)	(3)	(4)
1/1	1800	1710	1680	1650
1/2	2760	2790	2820	2430
1/4	4350	4140	4110	3870
1/8	6510	6630	6600	6150

Model: Parallel lines
 Design: Completely randomised
 Transformation: y' = log(y)
 Variance: Observed residuals
 Dilution step (Increasing): 2

Common slope(factor) = -0.276665
 Correlation |r|: 0.994347

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	0.0120923	0.0120923	19.949	0.000 (***)
Regression	1	1.47102	1.47102	>1000	0.000 (***)
Non-parallelism	1	3.90983E-08	3.90983E-08	0.000	0.994
Non-linearity	4	0.00236444	0.000591109	0.975	0.439
Standard	2	0.00221601	0.00110800	1.828	0.182
Sample 1	2	0.000148426	7.42132E-05	0.122	0.885
Treatments	7	1.48548	0.212211	350.084	0.000 (***)
Residual error	24	0.0145481	0.000606171		
Total	31	1.50003	0.0483879		

Sample 1			
Id.	11/184 in 1 ml water		
(iu/ml)	Lower limit	Estimate	Upper limit
Potency	3499.37	3736.28	3987.25
Rel. to Ass.	81.4%	86.9%	92.7%
Rel. to Est.	93.7%	100.0%	106.7%



Results for Collaborative Study CS473: Replacement of the WHO 1st International Standard for High Molecular Weight Urokinase, 87/594

A series of 4 independent assays has been requested, assaying the Test sample 11/184 against the Standard, 87/594 and. You are requested to return raw data to NIBSC. Raw data may be in the form of tables of end points (for example 50% lysis time) versus dose, or absorbance readings versus time. Where absorbance and time data are returned, please supply Excel spreadsheets (or Softmax platereader .pda files are acceptable). Please be sure to indicate the doses or dilutions of Standard and Test solutions and the arrangement of Standard and Test on the microtitre plate where appropriate. Example results sheets are provided and may be copied or you may generate your own if necessary.

Please return raw data (not the calculated potency of 11/184 versus 87/594).

Please fill in the table below to give an outline of the assay method used and provide information on dilutions and randomisation of sample preparation and measurement

Laboratory name and number	
What assay method did you use? (include information on source of reagents, e.g. plasma or fibrinogen; concentrations of thrombin, and plasminogen; nature of the end point; type of equipment)	
How many doses of urokinase did you include in each assay and how many replicates at each dose.	
Explain any randomisation you included in the layout of your experiments or order of measurements.	
Did you encounter any problems with the samples or assays	
Any other comments	

Please return results by email to Colin Longstaff. Colin.longstaff@nibsc.hpa.org.uk

Appendix 2

Acknowledgements to Participants

We are extremely grateful for the donation of the urokinase used to prepare the candidate IS, 11/184 and to the laboratories and personnel who gave their time and took part in the study to provide the data presented in this report. The following list is presented in a random order and is unconnected with the laboratory code specified in the report.

Chunming Rao Youxue Ding, Kai Gao, Lan Wang, Xinchang Shi Division of Biopharmaceuticals National Institutes of Food and Drug Control 2 Tiantan Xili Dongcheng District Beijing 100050 China	Dr. Nicola J Mutch School of Medicine and Dentistry Institute of Medical Sciences University of Aberdeen Foresterhill Aberdeen AB25 2ZD United Kingdom	Krasimir Kolev Semmelweis University Department of Medical Biochemistry 1094 Budapest, Tűzoltó u. 37-47. 1094 Hungary
Dr. Helen Philippou/Dr. Emma Smith Leeds University Division of cardiovascular and Diabetes research LIGHT Laboratories Clarendon Way Leeds LS2 9JT United Kingdom	Prof Robert Medcalf/Be'eri Niego Monash University Australian Centre for Blood Diseases Level 6, Burnet Building 89 Commercial Road Melbourne, Victoria 3004 Australia	Joost Meijers University of Amsterdam Dept. Experimental Vascular Medicine, G1-143 Academic Medical Center Meibergdreef 9 Amsterdam 1105 AZ The Netherlands
Dr. Ralph Pannell/Wendy Kung Vascular Laboratory 763D Concord Ave Cambridge, MA 02138 USA	Terenzio Ignoni Gentium SpA Piazza XX Settembre, 2 Villa Guardia Como, 22079 Italy	Jyoti Patel/Mr Ravishankar Prajapati Bharat Serums and Vaccines Ltd Plot No. K-27, Anand Nagar, Additional M.I.D.C., Ambernath (East), Maharashtra, India
Dr. Jonathan Foley University of Vermont 208 S Park Drive CRF, Room 233 Colchester VT 05446 USA	Dr. Sophie Plé/Natacha Tixier PX Therapeutics 7, parvis Louis Néel – BP 50 MINATEC/BHT-52B Grenoble Cedex 9 38040 France	Prof. Ann Gils Laboratory for Pharmaceutical Biology KULeuven Herestraat 49, box 824 3000-Leuven Belgium
Dr Tetsumei Urano Hamamatsu University School of Medicine Dept. of Medical Physiology 1-20-1, Handa-yama, Higashi-ku Hamamatsu, 431-3192 Japan	Colin Longstaff NIBSC Biotherapeutics Blanche Lane South Mimms Herts, EN6 3QG United Kingdom	

Appendix 3

Participants' Comments on the Report and updates on the Participants' Report

All participants, 14/14 (one of which was NIBSC) agreed with the conclusion made in the report that preparation 11/184 be proposed as the WHO 2nd International Standard for High Molecular Weight Urokinase with a potency of 3200 IU per ampoule.

Laboratories 2, 9 and 10 identified minor typographical errors or omissions in the text or tables, which have been corrected.

Laboratory 15 suggested that the table containing stability data after reconstitution was unclear as to the meaning of 100% activity for candidate 11/184 and the mannitol/sucrose formulation. This table, Table 4, has been re-written now to include potencies expressed in IU as well as a percentage of the starting value of 11/184.

Data were added to the current report on the stability and potency determinations for ampoules of 11/184 after 6 months' storage at elevated temperatures (now Table 3).

New data are now included in the current report with an estimate for the molar concentration of urokinase based on active site titration. This concentration is not intended for endorsement by WHO but the estimate may be included in the Instructions for Use for this IS, as this type of information is often requested for some applications where IU are not appropriate.

Appendix 4

ISTH/SSC Experts' Comments on the Report

Professor Ann Gils, Chair of the Fibrinolysis Subcommittee of the SSC distributed the Participants' report with their comments for further expert review to all Subcommittee co-chairs: Tetsu Urano; Nicola Mutch; Osamu Matsuo, Shirley Uitte de Willige, Paul Kim, Jonathan Foley and Craig Thelwell

In addition, Professor Gils sent the report to the following experts in the field: Roger Lijnen, Kees Kluft, Mario Colucci, Eduardo ANGLES-CANO, Paul Declerck, Dirk Hendriks, Joergen Jespersen, Peter Andreasen

Comments were received from all 7 Subcommittee co-chairs and 5 of the nominated experts. All comments are provided below, with responses where necessary (*in italics*)

Comments of co-chairs fibrinolysis subcommittee:

Reviewer 1: I believe that this report well represents the obtained results from the collaborative study and can be submitted as the final report.

Reviewer 2: As a participant of the study I have also passed my comments on the report of the new WHO International Standard for High Molecular Weight Urokinase to NIBSC. I believe the report to be an accurate representation of the results obtained and support the conclusions drawn. I agree that it should be submitted as a final report to the WHO.

Reviewer 3: I agree that this report is submitted to WHO.

Reviewer 4: I also do not have any comments, and agree that it should be submitted as a final report to the WHO.

Reviewer 5: Although there is the potential for a conflict of interest with my name cited as an author on the report, I was not directly involved with the collaborative study or potency assignment. My contribution was to determine the molar concentration of the candidate material by active-site titration.

(Dr Thelwell worked on a small aspect of the study, active site titration on the starting material, and was not involved in any other aspect of the potency assignment which is the primary matter under consideration by the ECBS)

If this poses no problem then please report that I agree with the proposed replacement and value assignment with no additional comments.

Reviewer 6: I have no additional comments and agree that this be submitted as the final report to the WHO.

Reviewer 7: Approved.

Comments of other experts to which the report was sent to:

Reviewer 1: I have reviewed the document and have no comments or suggestions and accept it with pleasure

Reviewer 2: I have read the report with great interest and found it exhaustive, clearly written and very informative.

Reviewer 3: I read carefully the report; my felicitations to the participating laboratories and the authors of the report from the NIBCS, for this well performed and written international collaborative study of great practical value for the scientific community. There are some point that deserve attention:

1. Neither in the Summary nor in the text is mentioned if the urokinase preparation is of the single-chain or of the two-chain type or which is the proportion of these types in the preparation. The only mention about purity is that the preparation is >95 % HMW urokinase. I suppose it is tc-uPA because it is called HMW urokinase. However, I believe it is important to clearly indicate so, as in the first case you have the zymogen form that is resistant to inhibition and this is important to know for test performed in a plasma milieu as was the case for laboratories 1, 6, 8 and 10.

This is potentially a valid point that can be clarified in the report. As the reviewer suggests, conventionally HMW urokinase is taken to be the two chain active form of the enzyme rather than the single form of the zymogen and we indicated that the starting material was > 95% HMW urokinase, which was stated in the manufacturer's certificate of analysis. We have now updated the section dealing with the starting material in the report to say "HPLC and SDS PAGE analysis showed the urokinase present to be >95% high molecular weight two chain form." on page 2 in the first paragraph of Materials (additional wording underlined here).

2. Calculation of the potency of urokinase. The geometrical mean was used instead of the mean or the median. A geometrical mean is often used when data cover several orders of magnitude or

for variables with a multiplicative effect (as in economics). Such is not the case of the present results. The collected data will be better defined by the arithmetical mean (3245) or the median (3176) if necessary. The median is very similar to the value (3169) found with the optional method.

As is usual practice, one of the authors of the report is a statistician at NIBSC (P.R.) and provides the following justification for the use of geometrical means over arithmetical means. Data were analysed using parallel line bioassay methods which gives an estimate of log relative potency. All combination of estimates was performed using a log scale as recommended by pharmacopoeial guidelines (e.g. Eur Ph and USP) and WHO guidelines, hence the use of geometrical means.

3. The molar concentration of urokinase. I have a concern with the provided value (380 nM) that is around 2/3 of the molarity by mass (555 nM). The urokinase used was a purified product obtained from urine and the starting solution contained 30 µg/mL of urokinase. One mL aliquots were freeze dried in the ampoules. The content of each ampoule was dissolved in 1 mL water, so the concentration of urokinase is always 30 µg/mL i.e. 555 nM. Disparity (30% difference) between the molar mass concentration and the molar activity would mean that either the product lost activity or that there is a problem with the titration experiment.

Indeed there does seem to be some disparity between the activity determined by active site titration and the concentration of urokinase stated in the stating material. This could be due to a number of factors, such as overestimate of the initial protein concentration, the presence of inactive urokinase, and underestimate of the concentration of active sites by the titration method. In reality it is likely that all these factors are present and may be additive. Since the main purpose of the study is to assign a biological activity to the Standard in IU, these additional parameters of urokinase protein concentration or concentration of urokinase active sites are only of secondary importance, and are given in the Instructions for Use as a guide for users of the Standard if they require additional information besides IU.

Some typographical or writing flaws:

Summary

p1 line 8. delete laboratories after "countries"

Introduction

p1 line 3. urokinase instead of "urokinse"

Materials

p2 line 2. "purified urokinase purified" delete one of those "purified"

All corrections have been made

Reviewer 4:

Comment 1: Specific activity of start material = therapeutic urokinase batch:

The batch urokinase that was provided by the manufacturer has a concentration of 11 mg/ml (203 µM) with a specific activity of > 100000 IU/mg protein. On page 12 it is indicated that the molar concentration of active urokinase is calculated to be 139 µM which is only 68% of the content. How can the activity of a therapeutic urokinase product can only be 68%?

This is the same concern raised above by Reviewer 3 in point 3, please refer to the response above.

Comment 2: Stability issue:

In table 3 it is mentioned that the urokinase activity in ampoules is very stable during storage with no degradation at 20°C, 37°C, 45°C and only 16% after 6 months at 56°C. However, in table 4: a loss of activity of **21.6%** was reported for the albumin formulation and a loss of activity of 75.6% for the mannitol/sucrose formulation after 4 days at 4°C. Can you explain this?

Table 3 and Table 4 deal with 2 different types of stability data: stability of sealed ampoules and freeze dried contents (Table 3), and the stability of urokinase solution after reconstitution of the contents of the ampoules (Table 4). The processes involved in loss of activity are different in these 2 situations.

Reviewer 5: Approved.



WHO International Standard
2nd International Standard for High Molecular Weight Urokinase
NIBSC code: 11/184
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The potency of the Standard was determined by International Collaborative Study and found to be 3200 IU per ampoule in comparison with the 1st International Standard for High Molecular Weight Urokinase (87/594) (1). The Standard was established by the Expert Committee on Biological Standardisation of the World Health Organisation (WHO) in October 2012. The potency of 3200 IU per ampoule was assigned using fibrin-based clot lysis assay methods. Caution is advised when interpreting results using alternative assay methods. This Standard is intended for use as an activity standard in enzymatic assays. Active site titration of the bulk material provides an estimate for active enzyme in the ampoule after filling of 390 µM, however this value is only an estimate from one laboratory and should be treated as a guide only. The Standard has not been calibrated as an antigen standard.

2. CAUTION

This preparation is not for administration to humans.

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

3. UNITAGE

3200 IU/ampoule

4. CONTENTS

Country of origin of biological material: United Kingdom, Italy.

5. STORAGE

Unopened ampoules should be stored in the dark at or below -20°C
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.

Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.

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

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

Each ampoule contains the residue after freeze drying of 1ml solution of 10mM Hepes buffer, pH7.4, containing 0.15 M NaCl and 5 mg human albumin This solution was filled into approximately 4700 ampoules with mean fill weight of 1.0099 g and inter-ampoule CV of 0.14%. After freeze drying the average residual moisture was 0.4%.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label. NIBSC follows the policy of WHO with respect to its reference materials.

Assays were performed on samples of 11/184 stored at elevated temperatures for 0.5 years and no significant loss of activity was observed at 4, 20, 37 and 45 °C. Further studies to assess long term stability are ongoing.

9. REFERENCES

1) P J Gaffney, A. B. Heath. A collaborative study to establish a standard for hlow molecular weight urokinase Thromb. Haemost. 64: 398-401 (1990)

10. ACKNOWLEDGEMENTS

Participation of the following laboratories in the International Collaborative Study to establish the potency of the 3rd International Standard for tissue plasminogen activator (98/714) is gratefully acknowledged.

11. FURTHER INFORMATION

Further information can be obtained as follows:

This material: enquiries@nibsc.hpa.org.uk

WHO Biological Standards:

<http://www.who.int/biologicals/en/>

JCTLM Higher order reference materials:

<http://www.bipm.org/en/committees/ijc/jctlm/>

Derivation of International Units:

http://www.nibsc.ac.uk/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx

Ordering standards from NIBSC:

http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx

NIBSC Terms & Conditions:

http://www.nibsc.ac.uk/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.hpa.org.uk

13. CITATION

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

14. MATERIAL SAFETY SHEET



Physical and Chemical properties	
Physical appearance: Freeze dried powder	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: Yes	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	Contains material of human origin
Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin
Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

above options shall constitute an entire discharge of the Institute's liability under this Condition.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: <i>United Kingdom</i>
* Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: <i>10 mg</i>
Toxicity Statement: <i>Toxicity not assessed</i>
Veterinary certificate or other statement if applicable.
Attached: <i>No</i>

15. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.

It is the responsibility of the Recipient to determine the appropriateness of the standards or reference materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependant on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.

All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.

The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.

The total liability of the Institute in connection with this agreement, whether for negligence or breach of contract or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.

If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the