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The certification of a European Reference Plasma for Factor VIII

BCR-629

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Report EUR 21061

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REFERENCE MATERIALS

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ABSTRACT

Assays of Factor VIII in clinical laboratories are subject to large variability and one of the sources of this variability is the wide range of reference plasmas used. An International Standard for Factor VIII in plasma is available from WHO but only in limited quantity. The aim of this project was to establish a European Reference Plasma for Factor VIII, carefully calibrated against the WHO standard in a large collaborative study, in order to improve quality control in European laboratories.

The European Reference Plasma for Factor VIII (BCR-629) consists of lyophilised normal human plasma, pooled from 17 donors, in sealed glass ampoules. Prior to pooling and ampouling the plasmas were centrifuged twice and buffered in order to enhance stability, according to previous studies.

BCR-629 has been calibrated for FVIII relative to the primary WHO International Standard for FVIII in plasma (3rd International Standard Factor VIII/vWF, plasma code 91/666) in a collaborative study involving 14 laboratories in 10 countries. There was good agreement between laboratories and between the 1-stage and chromogenic assay methods. Preliminary stability studies based on accelerated degradation predicted satisfactory long term stability for the standard when stored at –20 °C, but further monitoring of stability in real time will be performed.

The results of the calibration study indicated that preparation BCR-629 is suitable to serve as the European Reference Plasma for Factor VIII.

The assigned value for BCR-629 is as follows:

Material	*Mean potency*[1)] *(IU per ampoule)*	*Uncertainty*[2)] *(IU per ampoule)*	*No of accepted results*
BCR-629 Reference Plasma for Factor VIII	0.70	0.02	17

[1)] This result is the unweighted geometric mean from 17 independent estimates

[2)] The uncertainty is given as the half width of the 95% confidence interval of the mean defined in [1)]

LIST OF ABBREVIATIONS AND SYMBOLS

APTT	activated partial thromboplastin time
CV	coefficient of variation
EQAS	external quality assurance scheme
FVIII	coagulation factor VIII
FVIII:C	coagulation factor VIII: clotting activity
GCV	geometric coefficient of variation
HBsAg	Hepatitis B surface particle antigen
HCV	Hepatitis C virus
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HIV	human immune deficiency virus
ICSH	International Committee on Standards in Haematology
ISTH	International Society on Thrombosis and Haemostasis
IU	International Unit
SSC	Scientific and Standardisation Committee
vWF	von Willebrand factor
WHO	World Health Organisation

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1. INTRODUCTION

1.1 Background and need for a CRM

Testing of patients' blood samples for various coagulation indices is an important aspect of the diagnosis and treatment of disorders of coagulation. Assays of Factor VIII (FVIII) are mainly carried out in association with diagnosis and treatment of haemophilia A, caused by genetic deficiency of FVIII, but are also performed in relation to acquired coagulation defects and to epidemiological studies of normal individuals.

Coagulation measurements are generally recognised to suffer from large variability. This is partly because of the biological nature of the test samples and reagents, and partly because of the multiplicity of reagents available. Standardisation is exerted at international level, by the provision of reference standards by the World Health Organisation (WHO), co-ordinated through the Scientific and Standardisation Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) and the International Committee on Standards in Haematology (ICSH), and of reference methods and guidelines provided by the ISTH and ICSH. At Community level, laboratories have access to WHO standards, but these are only available in limited quantities and therefore need to be supplemented with national, local, and commercial standards. An additional level of national control is the operation of external quality assurance schemes (EQAS), in which results of different laboratories are compared.

Although an international plasma standard for FVIII has been available for factor VIII assays since 1981 (1, 2), considerable interlaboratory variability still exists, as can be judged from results from UK EQAS surveys, showing a wide distribution of results, with coefficients of variation (CV) of 20-30%. Analysis of results according to the various reference plasmas used indicates that the different reference plasmas are a major source of variability. This is also suggested by the fact that, in an international collaborative study, using a common reference plasma, interlaboratory CVs were less, around 11% (3). The availability of a European reference plasma would allow much more extensive calibration of commercial plasmas, and, unlike the WHO standard, could also be used for quality control in laboratories of member states.

1.2 Choice of Material

The most suitable material for a FVIII reference plasma is fresh normal plasma from a Blood Transfusion Centre, collected and treated in a manner designed to preserve the biological activity of FVIII (see also Section 4.1). A description of the actual material used and the method of processing is in Sections 4.2 and 4.3.

1.3 The International Unit for Factor VIII

The International Unit is defined as the amount of FVIII found in 1 mL of fresh, pooled normal human plasma. The original 1st International Standard Factor VIII/vWF plasma (code 80/511) was calibrated relative to freshly collected normal plasma pools comprising a total of 315 donors in a multi-centre collaborative study (1). In this study the local fresh plasma pools were arbitrarily assigned a unitage of 1 IU per mL. The 3rd International Standard Factor VIII/vWF, plasma (code 91/666) was calibrated in a study which allowed comparison with the previous 2nd International Standard and also freshly collected normal plasma pools to ensure continuity of the International Unit (3). A mean value of 0.80 IU per ampoule (95% confidence limits 0.78 - 0.83) was assigned to the 3rd International Standard based on the results from 14 laboratories. This was the WHO standard in use at the time of calibration of the BCR reference plasma; it has recently been replaced by the 4th International Standard (1998). The traceability of successive International Standards is shown in Figure 1.1.

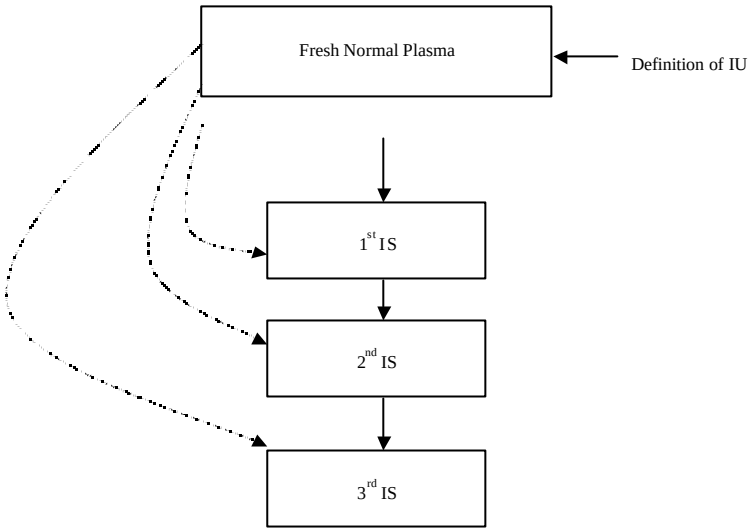

Figure 1.1 – Traceability scheme of the international unit (IU) for Factor VIII

1.4 Design of the Collaborative Study

The objective of the collaborative study was to assign a value in International Units (IU) to the proposed European reference plasma BCR-629 by assay relative to the existing WHO reference plasma for FVIII (3rd International Standard Factor VIII/vWF, plasma, code 91/666). Details of the protocol and methodology are given in Section 7.

1.5 Original Definition

One International Unit (IU) of Factor VIII is the amount in 1 mL of average fresh normal plasma, collected from a large number of doners in several laboratories. The first International Standard (IS) was calibrated by assay against fresh normal plasma from 315 donors. Subsequent International Standards were calibrated against the previous IS, and also checked against fresh normal plasma from a similar large number of donors to avoid the possibility of long-term drift of the International Unit.

2. PARTICIPANTS

2.1 Preparation and ampouling of materials

- National Institute for Biological Standards and Control, Potters Bar UK

2.2 Homogeneity and stability testing

- National Institute for Biological Standards and Control, Potters Bar UK

2.3 Certification measurements (14 laboratories, 10 countries)

- Department of Haemostasis, Finnish Red Cross Blood Transfusion Service, Helsinki FI
- Department of Clinical Chemistry and Blood Coagulation, Karolinska Hospital, Stockholm SE
- Department of Haematology, Royal Hallamshire Hospital, Sheffield UK
- Department of Transfusion Medicine and Coagulation, University of Freiburg, Freiburg DE
- Service d'Hematologie Biologique, Hopital Antoine Beclere, Paris FR
- Department of Haematology, University of Wales College of Medicine, Cardiff UK
- Department of Hematology, S Bortolo Hospital, Vicenza IT
- Haemostasis and Thrombosis Research Unit, University Hospital, Leiden NL
- Laboratoire d'Hemostase, Cliniques Universitaires Saint-Luc, Brussels BE
- Coagulation Unit, St James's Hospital, Dublin IR
- Regional Haemophilia Centre, The Royal Infirmary, Edinburgh UK
- Centro Emofilia e Trombosi "A Bianchi Bonomi", Isituto Medicina di Milano, Milano IT
- University Hospital Aarhus/Skejby, Haemophilia Centre and Coagulation Laboratory Aarhus DK
- National Institute for Biological Standards and Control, Potters Bar UK

2.4 Statistical analysis

- National Institute for Biological Standards and Control, Potters Bar UK

Participants for the certification exercise were selected on the basis of their known expertise and lengthy experience in FVIII assays and on the basis of their involvement and interest in standardisation and quality control. Several of the participants had previously taken part in international collaborative studies on FVIII.

3. PRELIMINARY STUDIES

FVIII reference plasmas for the United Kingdom and for WHO have been manufactured at NIBSC for over 20 years and previous studies have identified the important aspects of plasma processing necessary to preserve the biological activity of FVIII and produce a stable standard (1, 3, 4).

These are:

(a) use of fresh plasma rather than frozen;

(b) collection by centrifugation from whole blood rather than by plasmapheresis;

(c) use of a second centrifugation step to eliminate residual cells;

(d) buffering of plasma as soon as possible after collection.

No additional preliminary studies were therefore undertaken and these were the conditions used to collect and process the material for the European Reference Plasma for FVIII (BCR-629).

4. PREPARATION OF THE CANDIDATE REFERENCE PLASMA

4.1 Justification for the choice of material

Routine measurement of FVIII on patients' plasma samples is carried out on freshly collected citrated plasma. The ideal reference material should therefore consist of fresh citrated plasma. However, practical considerations of stability, transport and storage make the use of fresh or frozen plasma impossible, and all International and National reference plasmas for FVIII consist of lyophilised plasma.

NIBSC has over 20 years' experience in the production of stable reference plasmas for FVIII and therefore the procedures which were previously successful have been adopted (1, 3, 5).

4.2 Collection and processing of plasma

Fresh blood was collected on 3 August 1995 from 17 donors at the North London Blood Transfusion Centre, Colindale, UK, into citrate phosphate dextrose-adenine anticoagulant at a ratio of 63 mL anticoagulant to 450 mL blood. All units of plasma used to prepare the reference plasma were tested and found negative for Hepatitis B surface antigen, antibodies to Hepatitis C and antibodies to HIV 1 and 2. After the first centrifugation of 4200 rpm for 10 minutes at 22 °C, each unit of plasma was buffered by the addition of 10 mL 1.0 mol/L HEPES, giving a final HEPES concentration of approximately 0.05 mol/L, then centrifuged again under the same conditions. After overnight storage at 4 °C the individual donations were pooled in a large filling vessel with constant stirring, and filled into approximately 3,600 glass ampoules.

4.3 Ampoule filling and freeze-drying

Ampoule filling, freeze-drying and secondary desiccation were carried out according to the conditions used for the preparation of International Biological Standards (5). The candidate reference plasma was filled on 4 August 1995 into approximately 3,700 transparent glass ampoules, coded 95/540, at a volume of 1.0 mL per ampoule. Filling was carried out at a temperature of 4 °C. Check weighing was carried out at frequent intervals during the fill to check for constancy of fill volume (see section 5.1) and the filling sequence was identified by numbering of ampoules. The freeze-drying cycle of 5 days was followed by secondary desiccation over phosphorous pentoxide for 6 days before the ampoules were sealed under dry nitrogen and stored at –20 °C. The number of ampoules taken into stock was 3,689.

5. TESTING OF HOMOGENEITY AND STABILITY

5.1 Homogeneity Tests

5.1.1 Check weights during ampoule filling

Check weight ampoules were included at every 50th position in the fill sequence. The balance used for measuring the check weights was calibrated with an accuracy of ± 0.1 mg for a 1.0 g standard checkweight by an organisation accredited by the United Kingdom National Measurement Accreditation Service. The criterion of acceptability for filling plasma standards is an overall CV of less than 0.75%, this is checked on a rolling basis during the fill.

The mean content of 76 ampoules checked during filling was 1.0307 g, with a range of 1.0171 g to 1.0541 g and a CV of 0.61% and 95% confidence limits of ± 1.22%. Individual check weight values are given in Table 5.1 and shown graphically in Figure 5.1.

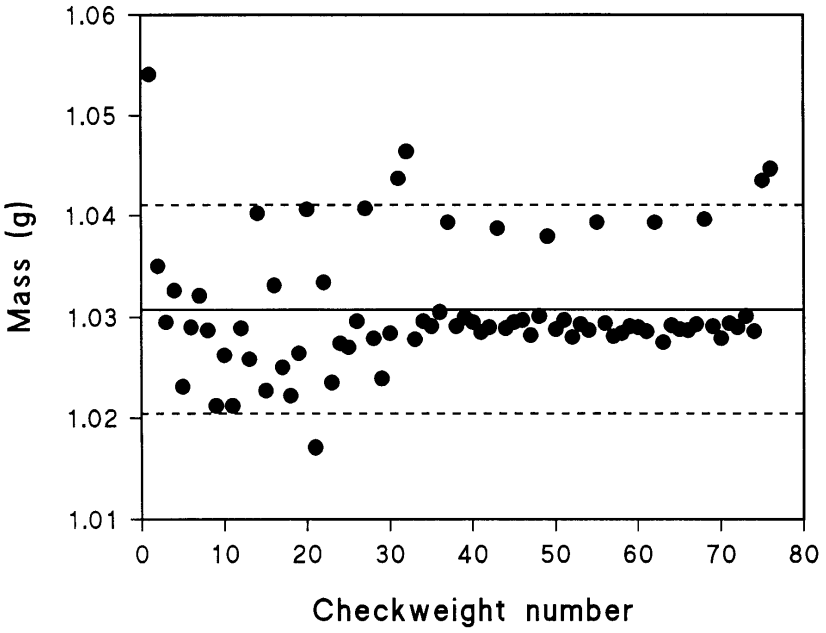

Figure 5.1 - Check weights during ampoule filling.

Lines indicate the mean mass (_____) and +/- 1% of the mean mass (------)

Table 5.1 - Check weights during ampoule filling of BCR-629

Checkweight no	*Weight (g)*	*Checkweight no*	*Weight (g)*	*Checkweight no*	*Weight (g)*	*Checkweight no*	*Weight (g)*
1	1.0541	21	1.0171	41	1.0285	61	1.0286
2	1.0350	22	1.0334	42	1.0290	62	1.0393
3	1.0295	23	1.0235	43	1.0387	63	1.0275
4	1.0326	24	1.0274	44	1.0289	64	1.0292
5	1.0231	25	1.0270	45	1.0295	65	1.0288
6	1.0290	26	1.0296	46	1.0297	66	1.0287
7	1.0321	27	1.0407	47	1.0282	67	1.0293
8	1.0287	28	1.0279	48	1.0301	68	1.0396
9	1.0212	29	1.0239	49	1.0379	69	1.0291
10	1.0262	30	1.0284	50	1.0288	70	1.0279
11	1.0212	31	1.0437	51	1.0297	71	1.0294
12	1.0289	32	1.0464	52	1.0280	72	1.0290
13	1.0258	33	1.0278	53	1.0293	73	1.0301
14	1.0402	34	1.0296	54	1.0287	74	1.0286
15	1.0227	35	1.0291	55	1.0393	75	1.0435
16	1.0331	36	1.0305	56	1.0294	76	1.0447
17	1.0250	37	1.0393	57	1.0281		
18	1.0222	38	1.0291	58	1.0284		
19	1.0264	39	1.0300	59	1.0291		
20	1.0406	40	1.0295	60	1.0290		

5.1.2 Mass

The mass of the freeze-dried plasma was estimated in six randomly selected ampoules. The mass was obtained by subtracting the mass of the washed and dried empty ampoule from the mass of the ampoule containing the freeze-dried plug (Table 5.2). Mean content of 6 ampoules was 85.4 mg, with a range of 84.6 mg to 86.0 mg and a coefficient of variation of 0.74%. The measured inter-ampoule variability also includes variability introduced during the washing and drying of the ampoules and is therefore probably an overestimate.

Table 5.2 - Masses of 6 ampoules of the freeze-dried candidate reference plasma BCR-629

Ampoule N°	*Total mass (g)*	*Mass of empty*	*Mass of Material*
1	6.1915	6.1055	0.0860
2	6.2132	6.1273	0.0859
3	6.2157	6.1310	0.0847
4	6.2268	6.1409	0.0859
5	6.1531	6.0678	0.0853
6	6.2280	6.1434	0.0846
Mean			0.0584
Standard Deviation			0.00632
CV			0.74 %

5.1.3 APTT testing

Since assay methods for Factor VIII have CVs in the range 5% to 10% these are not suitable for homogeneity testing. It was therefore decided to adopt a different test, which has reduced variability, the activated partial thromboplastin time (APTT) (6). The APTT forms the basis of the one-stage FVIII clotting method in that the end-point clotting time is dependent on the same coagulation pathway. The APTT test result is an absolute measurement of clotting time and is therefore not calibrated relative to any reference material. For the homogeneity testing APTT measurements were carried out on 21 randomly selected ampoules and also on 21 identical aliquots of a frozen normal plasma. The results are shown in Table 5.3. Results from the tests on the identical aliquots of frozen normal plasma indicate that the variability of the method (GCV%) is 0.97% (n=21). The inter-ampoule variability of BCR-629 (GCV 0.80%) was slightly less than the variability between the identical aliquots of the frozen normal plasma (GCV 0.97%). The fact that the CV for BCR-629 is no greater than that of the identical frozen aliquots indicates no detectable inhomogeneity in BCR-629 by this method.

Table 5.3 - APTT measurements on the candidate reference plasma BCR-629

*Mean APTT values for ampoules of BCR-629**		*Mean APTT values for frozen aliquots**	
33.3	33.2	32.4	32.5
33.0	33.1	33.1	32.7
32.8	33.4	32.7	33.1
33.6	33.3	33.1	32.3
33.6	33.0	32.7	32.6
33.5	33.3	32.9	32.6
33.4	33.2	32.3	32.4
33.6	33.3	32.6	33.0
33.6	33.8	33.1	32.3
33.0	33.7	32.8	33.4
33.2		32.8	
Mean APTT (seconds)	33.3	32.7	
Range	32.8 - 33.8	32.3 - 33.4	
GCV%	0.80	0.97	
95% conf Limits	33.2 - 33.4	32.6 - 32.9	

*Each APTT estimate is the mean from three replicate determinations on each ampoule or frozen aliquot.

5.2 Stability Testing

5.2.1 Residual moisture

Residual moisture was determined by Karl Fischer titrimetry with an uncertainty value (CV) of 5%; standardisation of the moisture meter was achieved using a check solution, provided by a UKAS accredited organisation, containing a known amount of water in methyl cellulose. Previous experience indicates that long-term stability of biological reference preparations requires low residual moisture. In order to achieve low residual moisture the candidate reference plasma, BCR-629, underwent both freeze-drying and secondary desiccation. Residual moisture was determined on three randomly selected ampoules after freeze-drying and on another three ampoules after completion of both freeze-drying and secondary desiccation. The levels of residual moisture given in

Table 5.4 (<0.2%) are similar to those found with other International Reference Plasmas prepared under the same conditions and indicate satisfactory processing of the candidate plasma preparation.

Table 5.4 - Residual moisture as mass fraction % in the candidate reference plasma BCR-629 after freeze-drying (FD) only and after both freeze-drying and secondary desiccation (FD+SD)

Sample No	*Moisture %*	
	FD Only	FD + SD
1	0.67	0.18
2	0.70	0.15
3	0.75	0.17
Mean	0.70	0.17

5.2.2 Accelerated degradation study

5.2.2.1 Design of the study

Estimates of the stability of the candidate reference plasma BCR-629 have been obtained from accelerated degradation studies. Ampoules of the candidate reference plasma, BCR-629, were stored at elevated temperatures (+4 ,+20 ,+37 ,+45 ^{o}C) for various periods before the estimation of potency relative to ampoules stored at –20 ^{o}C. This data has been used to predict the stability of the candidate reference plasma BCR-629 when stored at the bulk storage temperature of –20 ^{o}C from the Arrhenius equation (7). Potency estimation was carried out using the one-stage clotting method for FVIII with a variability (GCV) of 5%. The stability study was carried out in two exercises - a short-term study with samples tested after 3 and 6 months storage and a longer term study with samples tested after 33 months storage.

5.2.2.2 Results and discussion

Sampling times together with the residual potency of samples stored at elevated temperatures are given in Table 5.5. Predicted degradation rates are given in Table 5.6. For ampoules stored at –20 ^{o}C (bulk storage temperature) the predicted losses were 0.013 and 0.102% loss per year from the short- and long-term studies, respectively. The result obtained from the long-term storage should be considered a more reliable estimate since this has been extrapolated from degradation measured at temperatures to which the candidate reference plasma BCR-629 may be exposed during transport and use albeit transiently. These values are typical for those found with other reference plasmas for FVIII and, indicate that the candidate reference plasma BCR-629 is suitably stable for long-term use when stored at –20 ^{o}C. The results also indicate that, for most dispatches within Europe, special arrangements to conserve temperature during mailing should not be necessary.

Table 5.5 - Residual potency of accelerated degradation samples

Storage time	*Residual potency (% of –20 °C ampoules)*			
(months)	+4	+20	+37	+45
0.5	-----	-----	-----	94, 78, 82, 87
3	-----	-----	60, 61, 61	26, 27, 33
6	101, 104, 97, 105	97, 90, 87, 88	54, 59, 39, 39	-----
33	90, 91, 91, 95	83, 86, 79, 81	40, 50, 31, 31,	-----

Results are single estimates of FVIII from individual ampoules

Table 5.6 - Predicted degradation rates for the candidate reference plasma BCR-629 based on the short-term (3 and 6 months storage) and long-term accelerated degradation study (33 months)

Storage temperature (°C)	*Predicted degradation rates (% loss per year)*	
	Short-term study (3 and 6 months storage)	Long-term study (33 months storage)
-20	0.013	0.102
+4	1.145	1.606
+20	14.002	7.650

5.2.3 Real-time stability studies

Real-time stability of the candidate reference plasma was investigated by comparing estimates of FVIII obtained in September 1999 with estimates obtained in the same laboratory (study code 9) during the original calibration exercise in December 1995. Estimates of FVIII, at both times, were carried out on 4 ampoules according to instructions distributed to the participants using the same methodology (chromogenic method), instrumentation (ACL 3000 plus) and reference preparation (3rd International Standard FVIII/vWF plasma 91/666).

There was no significant difference (unpaired t test) between the FVIII estimates from both time points (data not shown). As a further check on long-term stability it is recommended that samples of the reference plasma stored at –20 °C be assayed against samples stored at –70 °C.

6. CERTIFICATIONS MEASUREMENTS

6.1 Principle of calibration

Calibration of the candidate reference plasma BCR-629 involved the estimation of FVIII relative to the established primary reference preparation for FVIII in plasma (WHO 3rd International Standard Factor VIII/vWF plasma, 91/666). This standard has an assigned value of 0.80 IU per ampoule which is traceable to the original definition of the International Unit in fresh pooled normal plasma (see section 1.3).

6.2 Materials

The following materials were included in the study, coded as indicated:

A 3rd IS Factor VIII/vWF Plasma (91/666)

B Proposed European Reference Plasma BCR-629

H House Standard (where available)

Four ampoules of A and B were provided by NIBSC for each laboratory, with one or 2 additional sets of 4 ampoules for laboratories performing 2 and 3 methods, respectively. House standards were provided locally, and were either commercial reference plasmas or local plasma pools - all had been calibrated against the 3rd IS Factor VIII/vWF plasma (91/666) and were included so that laboratories could check their local calibration procedure.

6.3 Assay design

The participants were asked to follow one of 3 assay designs depending on the number of samples they were able to include in a single assay. Each design involved the performance of 4 independent assays of 6, 5 or 4 samples. For each sample at least 3 different dilutions of each plasma were requested. The number of days for the assays was not specified as each assay is independent, but in practice most laboratories performed the assays over more than one day.

Within an individual assay one, 2 or all 3 (where 4, 5 or 6 samples were included) of the preparations were repeated which enabled a measure of the variability within the assay to be obtained. Participants were requested to use their normal in-house validated one-stage, two-stage or chromogenic method for FVIII:C.

6.4 Analytical methods

Some laboratories performed more than one method, giving a total of 19 data sets, each of which was analysed individually. A description of the methods used by each laboratory is given in Table 6.1. Twelve laboratories performed the one-stage clotting method. This method is very similar to the APTT used in the homogeneity test (section 5.3). The APTT measures the clotting time of normal plasma in the presence of activators (APTT reagent) whereas the one-stage clotting method for FVIII measures the ability of added FVIII (test sample) to shorten the clotting time of a FVIII-deficient plasma in the presence of activators. Participants used a wide variety of APTT reagents, FVIII-deficient plasmas and instrumentation.

Three laboratories performed the two-stage method based on the principle that the first-stage incubation mixture produces an amount of the enzyme factor Xa which is proportional to the amount of FVIII in the mixture; the amount of factor Xa is then estimated in the second stage by the clotting time on addition of normal plasma.

Four laboratories performed the chromogenic method, using commercial kits from either Chromogenix or Immuno. This method is based on the same principle as the two-stage method,

except that more purified reagents are used in the first stage and the amount of FXa generated is estimated by chromogenic substrate assay.

Table 6.1 - Assay methods used in the collaborative study

Lab. no.	*one-stage (a)*			*two-stage (b)*		*chromogenic (c)*	
	reagent	*def-plasma*	*instrument*	*reagents*	*instrument*	*reagents*	*instrument*
1	IL	IL	ACL	----	----	----	----
2	IL	Local SHP	ACL	----	----	----	----
3	IL	IL	ACL	----	----	----	----
4	Organon	local SHP	MLA	----	----	----	----
5	----	----	----	in house	ACL	----	----
6	Dade	Dade	S&G	Immuno	S&G	Immuno	microtitre
7	Organon	local SHP	ACL	----	----	Chromogenix	microtitre
8	----	----	----	----	----	Chromogenix	Cobas Mira
9	IL	Organon	ACL	in house	ACL	Chromogenix	ACL
10	Diagen	Immuno	Sysmex	----	----	----	----
11	Organon	Organon	Hemolab	----	----	----	----
12	Dade	Dade	ACL	----	----	----	----
13	Dade	Local SHP	MLA	----	----	----	----
14	Stago	Stago	Stago	----	----	----	----

Key to abbreviations:
IL - Instrumentation Laboratory; ACL - IL Automated Coagulation Laboratory
S&G - Schnitger and Gross coagulometers; SHP - severe haemophilic plasma
MLA – Medical Laboratory Analyser

6.5 Statistical analysis of the data

From the measurements of clotting times or optical densities of individual dilutions of the test plasma, potencies in each assay were calculated by comparative bioassay against the 3rd International Standard Factor VIII/vWF plasma (91/666), using well-established statistical methods based on parallel line analysis (8). This analysis relies in the comparison of linear and parallel dose-response relationships for the standard and test materials. Since an extreme range of dilutions of assay samples gives a sigmoid dose-response relationship, it is sometimes necessary to omit one or more responses from either end of the dilution range in order to achieve a linear dose-response.

For the majority of laboratories the untransformed response against the log of the dose was considered. For the 3 laboratories using the chromogenic assay method (6c, 7c and 9c) and also for laboratory 10a, the log transformation of the response was used to ensure parallelism and linearity. For 3 laboratories it was necessary to omit some of the observations to achieve parallelism and linearity as detailed:

- Lab 6a - for assay 2 the result for the highest dose of preparation A is omitted,
- Lab 6b - for assay 1 the results for preparation B are omitted,
- Lab 7c - the results for assay 1 are omitted.

After the above transformations and amendments the assumptions of parallelism and linearity held in 96% and 85% of cases, respectively. Where the assumptions still did not strictly hold the irregularities were not considered severe enough to require any further changes to be made.

Laboratory 8 reported results as potencies of single dilutions against an external standard and therefore no statistical analysis could be performed. Potencies were calculated but not included in the overall means.

Combined potency estimates were calculated for each laboratory across all assays by taking the geometric mean of the results from all assays. Potency estimates by method and overall were obtained by taking the appropriate geometric means of the laboratory means.

The geometric coefficient of variation (GCV%) provided a measure of the variability within and between laboratories (9).

6.6 Results of the calibration

Table 6.2 shows the potency estimates together with 95% confidence limits and GCVs in individual laboratories for the candidate reference plasma BCR-629. There was good agreement within laboratories with all GCVs less than 9% except for laboratory 9a where the individual assay results were highly variable. Since this data set was atypical in its variability it was not included in the overall means. Inclusion or exclusion of the data from laboratory 9a did not affect the overall mean value of 0.70 IU per mL. The potency estimates are also shown graphically in the histogram (Figure 6.1).

Table 6.3 shows the estimated potencies of the proposed European Standard according to the assay technique used. The results show that the one- and two-stage methods give very comparable results while that obtained when the chromogenic method is used is somewhat lower.

The potency estimates for the proposed European Standard for the individual laboratories range from 0.65 to 0.77 IU per ampoule. The overall potency estimate across all the laboratories is 0.70 IU per ampoule with a GCV of 4.83% and 95% confidence limits of 0.68 - 0.72 IU per ampoule.

Table 6.2 - Estimated mean potencies in IU per ampoule, from individual laboratories, for the candidate reference plasma BCR-629 with 95% confidence limits and GCV.

Laboratory	*Individual potency estimates (IU/ampoule)*	*Mean Potency (IU/ampoule)*	*95% Confidence Limit*	*GCV (%)*
1a	0.65, 0.70, 0.68, 0.68	0.67	0.64 - 0.71	3.04
2a	0.84, 0.74, 0.78, 0.71	0.77	0.69 - 0.85	7.16
3a	0.73, 0.72, 0.80, 0.79	0.76	0.70 - 0.83	5.65
4a	0.78, 0.68, 0.73, 0.66	0.71	0.63 - 0.80	7.65
5b	0.72, 0.71, 0.68, 0.71	0.71	0.68 - 0.73	2.15
6a	0.66, 0.69, 0.73, 0.68	0.69	0.65 - 0.74	4.73
6b	0.70, 0.72, 0.72	0.72	0.69 - 0.75	1.72
6c	0.64, 0.68, 0.76, 0.65	0.68	0.60 - 0.77	8.10
7a	0.73, 0.71, 0.68, 0.67	0.70	0.66 - 0.74	3.79
7c	0.68, 0.62, 0.71	0.67	0.56 - 0.80	7.29
9b	0.68, 0.81, 0.72, 0.79	0.75	0.66 - 0.85	8.52
9c	0.65, 0.68, 0.59, 0.68	0.65	0.59 - 0.72	6.80

Laboratory	*Individual potency estimates (IU/ampoule)*	*Mean Potency (IU/ampoule)*	*95% Confidence Limit*	*GCV (%)*
10a	0.68, 0.67, 0.70, 0.66	0.68	0.65 - 0.70	2.43
11a	0.69, 0.70, 0.67, 0.70	0.69	0.67 - 0.71	1.79
12a	0.70, 0.67, 0.79, 0.73	0.72	0.65 - 0.81	7.27
13a	0.72, 0.67, 0.67, 0.66	0.68	0.64 - 0.73	4.13
14a	0.69, 0.67, 0.73, 0.63	0.68	0.62 - 0.75	6.07

Key to methods: a - one stage, b - two stage, c - chromogenic

N.B. data from laboratories 8 and 9a are not included for reasons given in the text

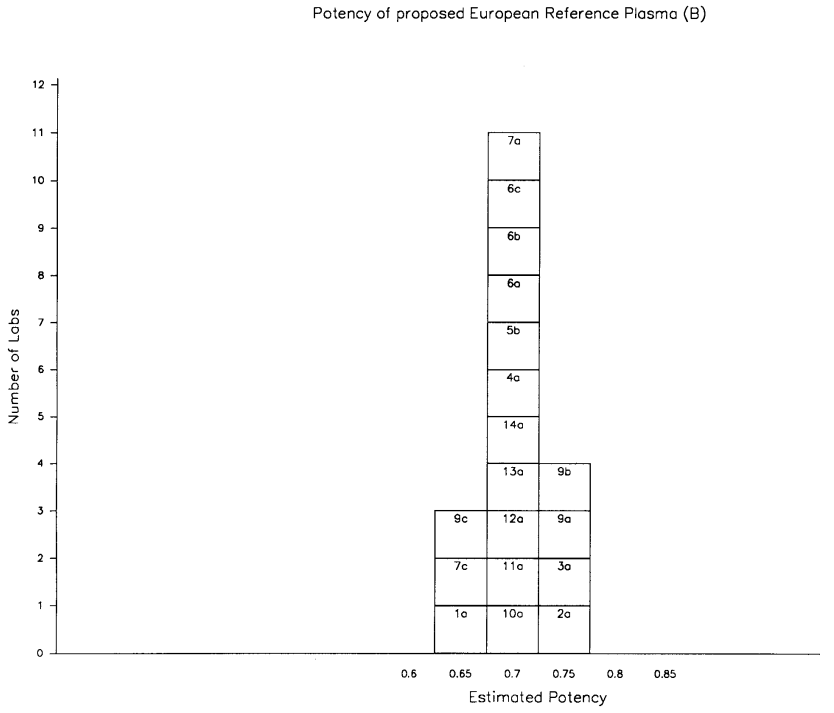

Figure 6.1 - Mean potency estimates (IU per ampoule) from individual laboratories for the candidate reference plasma BCR-629

Table 6.3 - Estimated potencies in IU per ampoule with GCV and 95% confidence limits according to the assay method used

Method	*Mean Potency (IU per ampoule)*	*95% confidence limits*	*GCV (%)*	*n*
One-stage (a)	0.70	0.68 - 0.73	4.73	11
Two-stage (b)	0.73	0.68 - 0.78	2.89	3
Chromogenic (c)	0.67	0.63 - 0.71	2.33	3
Overall	0.70	0.68 - 0.72	4.83	17

Table 6.4 shows the estimated potencies for the laboratory house standards, together with confidence limits and GCVs. Where the laboratory provided a potency for their House Standard these are also included.

Table 6.4 - Estimated potencies in IU per ampoule for the laboratory house standards with 95% confidence limits, GCVs and the potency value supplied by the laboratory where given

Laboratory	*Estimated Potency (IU/ampoule)*	*Laboratory Potency value (IU/ampoule)*	*95% confidence limit*	*GCV (%)*
1a	0.79	-	0.74 - 0.83	3.79
2a	1.06	1.10	0.92 - 1.21	8.78
3a	0.98	-	0.95 - 1.02	2.40
4a	0.97	-	0.86 - 1.08	7.59
5b	0.65	-	0.60 - 0.71	5.26
6a	0.58	0.6	0.55 - 0.62	3.80
6b	0.62	0.6	0.59 - 0.65	3.14
6c	0.61	0.6	0.60 - 0.63	1.84
7a	1.03	1.05	0.98 - 1.07	2.63
7c	1.15	1.00	1.01 - 1.29	4.96
9a	0.63	0.63	0.48 - 0.82	18.01
9b	0.70	0.63	0.62 - 0.79	7.99
9c	0.58	0.63	0.55 - 0.61	3.55
10a	0.76	-	0.70 - 0.83	5.49
11a	0.92	-	0.84 - 1.00	5.55
12a	0.78	-	0.68 - 0.88	8.41
13a	1.02	-	0.90 - 1.15	7.88
14a	0.86	-	0.80 - 0.94	5.37

6.6 Technical discussion

The level of agreement on FVIII assays of the proposed European Standard compares favourably with that found in other collaborative studies (1, 3, 4), both within and between laboratories. The variability between assays (intra-laboratory) within the individual laboratories was generally low with all except one laboratory having GCVs below 9% and eight laboratories having a GCV below 5% (Table 6.2). Agreement between laboratories (inter-laboratory) was very good, with a GCV below

5% for the overall mean value (Table 6.3).

Differences between methods were not statistically significant (t-test, $p>0.05$). One phenomenon which can cause assay discrepancies is activation of FVIII, which gives rise to elevated values in the 1-stage method, but not in the 2-stage or chromogenic methods. The fact that the one-stage potency was slightly higher than that by the chromogenic method is unlikely to indicate activation of FVIII, since the two-stage assay gave the same potency as the one-stage method, and it is more likely that these small differences arise by chance.

Potencies of the laboratories' house standards mostly agreed well with those already assigned, where these figures were available (Table 6.4), indicating that calibration of the house standards had been performed accurately.

As the results of the study were quite straightforward, it was not considered necessary to hold a meeting of participants. There were no critical comments on the report of the study and all participants agreed that the candidate reference plasma BCR-629 was suitable to serve as the European Reference Plasma for FVIII.

7. CERTIFIED VALUES AND UNCERTAINTIES

On the basis of the results of the collaborative study and with the agreement of the participants, the following certified values are obtained for reference plasma BCR-629:

Table 7.1 – Certified values for BCR-629

Material	*Mean potency*[1)] *(IU per ampoule)*	*Uncertainty*[2)] *(IU per ampoule)*	*No of accepted results*
BCR-629 Reference Plasma for Factor VIII	0.70	0.02	17

[1)] This result is the unweighted geometric mean from 17 independent sets of results estimates

[2)] This value is the half width of the 95% confidence interval the mean value in [1)]

8. INSTRUCTIONS FOR USE

8.1 Caution

This preparation is not for administration to humans. Each individual plasma donation and the final freeze-dried reference preparation was tested for HBsAg and for antibodies to HCV and HIV 1 and 2 and found to be negative. However, as with all preparations of human origin, this material cannot be assumed to be free from infectious agents. Suitable precautions should be taken in the use and disposal of the ampoule and its contents.

8.2 Description of samples

The FVIII reference plasma consists of glass ampoules (labelled BCR-629) containing approximately 1 mL aliquots of pooled fresh human plasma, that is buffered by the addition of N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) (1.0 mol/L pH 7.4) to a final concentration of 0.05 mol/L and freeze-dried under conditions used for International Biological Standards (1). Ampoules were filled with nitrogen gas before sealing.

8.3 Storage and reconstitution

Unopened ampoules should be stored at –20 ^{o}C or below. Allow ampoules to equilibrate to room temperature before opening. Reconstitute the total contents of each ampoule of reference plasma with 1.0 mL of distilled water. Do not attempt to weigh out any portion of the freeze-dried material. Swirl gently to dissolve the contents and transfer to a plastic tube. Store at room temperature during the period of the assay which should be within 2 hours of reconstitution.

8.4 Potency estimation of test samples

The plasma should be used as a reference material to determine the FVIII:C potency of test samples. At least 3 dilutions of the reference plasma should be made and a standard curve constructed. The potency of the test sample, also preferably at 3 dilutions, should be determined by parallel line analysis or by direct interpolation from the standard curve.

8.5 Guidelines for use of BCR-629 in quality control

The reference plasma is intended to be used for a number of purposes:

a) To check commercial reference plasmas for accuracy.

b) To check local plasma pools from time to time during their shelf life.

c) To give a reference value to NEQAS samples - for this purpose it is recommended that a small panel of laboratories (6-8) be used.

d) As a reference sample of known potency when a new method, or new reagents, are introduced.

In general it is recommended that for these purposes 2 assays on each of 2 ampoules (minimum) are performed. Criteria for acceptance of results should be based on local internal quality control procedures. The CRM is not intended for use as a working standard, ie for direct assay of patients' samples.

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Abstract

Assays of Factor VIII in clinical laboratories are subject to large variability and one of the sources of this variability is the wide range of reference plasmas used. An International Standard for Factor VIII in plasma is available from WHO but only in limited quantity. The aim of this project was to establish a European Reference Plasma for Factor VIII, carefully calibrated against the WHO standard in a large collaborative study, in order to improve quality control in European laboratories.

The European Reference Plasma for Factor VIII (BCR-629) consists of lyophilised normal human plasma, pooled from 17 donors, in sealed glass ampoules. Prior to pooling and ampouling the plasmas were centrifuged twice and buffered in order to enhance stability, according to previous studies.

BCR-629 has been calibrated for FVIII relative to the primary WHO International Standard for FVIII in plasma (3rd International Standard Factor VIII/vWF, plasma code 91/666) in a collaborative study involving 14 laboratories in 10 countries. There was good agreement between laboratories and between the 1-stage and chromogenic assay methods. Preliminary stability studies based on accelerated degradation predicted satisfactory long term stability for the standard when stored at –20 ^{o}C, but further monitoring of stability in real time will be performed.

The results of the calibration study indicated that preparation BCR-629 is suitable to serve as the European Reference Plasma for Factor VIII.

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