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COMMEMORATIVE ARTICLE

Laboratory testing and standardisation

T. W. BARROWCLIFFE
Formerly National Institute for Biological Standards and Control [NIBSC], Potters Bar, UK

Introduction

I joined National Institute of Biological Standards and Control (NIBSC) in 1974, but my introduction to the world of haemophilia had started some 8 years earlier, during my studies for a chemistry degree at Oxford University, when I met some patients with haemophilia during a short stay in the Churchill Hospital. I was impressed by the fortitude of these young patients who had had their lives severely disrupted by the disease, and intrigued to hear how little was known about their missing clotting factor, Factor VIII (FVIII). After I finished my degree I determined to pursue a career in coagulation, and eventually in 1969 I enrolled for a PhD at the Royal Free Hospital, under Katharine Dormandy.

As a chemist I knew nothing about the intricacies of clotting tests and assays, and had to undertake a crash course. I soon found out that measurements in coagulation did not have the same degree of certainty as those in chemistry, and that for assays of clotting factors, a standard of known activity was needed. The standard in use in the lab at that time was porcine FVIII, so the cry was frequently heard – 'pass the pig'! I did not understand why porcine FVIII was used as a standard, but I gathered after a while that it was more stable than human FVIII, and it was conveniently produced as a freeze-dried powder with a labelled potency. Quite how the number on the bottle was arrived at it never occurred to me to ask, but I would eventually find out when I left the Royal Free 5 years later and started my career in standardization at NIBSC.

Development of clotting assays

The partial thromboplastin time (PTT), in which clotting times were measured in the presence of platelets and the absence of tissue factor, was introduced in the 1950s. A major improvement was the introduction of phospholipid as a substitute for platelets [1], and a further refinement was the use of kaolin to provide reproducible activation of the contact factors [2] – the test then became known as the activated partial thromboplastin time (APTT). This test became the cornerstone for diagnosis of haemophilia and allied bleeding disorders, but could not distinguish between haemophilia A and B, as well as the rare bleeding disorders. Various studies showed considerable variability in sensitivity to the FVIII defect with different reagents [3,4], also it had a poor correlation with the severity of disease and was unsuited to monitoring the effect of treatment.

There was clearly a need for a quantitative assay of FVIII, and the first and simplest such assay to be published, in 1953, was the one-stage method developed by Dr Langdell in the laboratory of Kenneth Brinkhous at Chapel Hill [4]. This consisted simply of adding dilutions of the test sample to haemophilic plasma and measuring the PTT or APTT; the degree of shortening of the clotting times is proportional to the amount of FVIII in the sample, and by comparison with a standard material of known FVIII content (see subsequent section), the potency of the test sample can be calculated. A detailed review of the technical aspects of the one-stage method is given by Over [5]. The same principle of using deficient plasma as a substrate and measuring the shortening of the APTT was subsequently used to develop assays of factor IX and other intrinsic clotting factors [6]. The one-stage assay remains the most commonly used method and has changed little over the years; the use of artificially depleted deficient plasma and combined phospholipid/activator reagents, suitable for automation, have been the two main technical developments.

At the same time as this method was being developed, Dr Rosemary Biggs and colleagues at the MRC Blood Coagulation Research Unit in Oxford were working on a quite different method, the two-stage assay of FVIII. This arose from their researches into the mechanism of prothrombin activation in the absence of tissue factor; they realized that FVIII combined with activated FIX (FIXa), phospholipid and calcium ions to form 'intrinsic thromboplastin', which activated FX to FXa, the enzyme which converts prothrombin into thrombin [7].

Thus, in the first stage of their method dilutions of the FVIII containing sample were incubated with a source of FIXa, FX, phospholipid and Ca^{2+} ions, in the absence of prothrombin. Subsamples were then taken and added to a source of prothrombin and fibrinogen (usually normal plasma), and clotting times were measured after addition of Ca^{2+} ions. In the original method, published in 1955 [8], platelets were used as the source of phospholipid, but as with the

one-stage method this was soon replaced by a stable freeze-dried phospholipid reagent. Other technical variations were introduced over time, and these are reviewed elsewhere [9].

Detailed comparisons of the one-stage and two-stage methods have been published elsewhere [10]. Briefly, the one-stage method is simpler and easier to automate, but has a large variety of reagents (FVIII-deficient plasma and APTT reagents), which perhaps accounts for the fact that it is less precise. The two-stage method has less variation in reagents, which may explain why it is generally more precise, but is technically more complex and more difficult to automate. The latter problem has been circumvented by the introduction of chromogenic substrates to measure the FXa produced in the first stage, and the chromogenic version has now largely replaced the original clotting method. Unlike the one-stage assay, the two-stage method does not require a source of FVIII-deficient plasma, a distinct advantage to control laboratories such as NIBSC and to manufacturers of concentrates. The one-stage assay remains the most popular in clinical laboratories, but the chromogenic method is used extensively by manufacturers and control laboratories, and is the official method of the European Pharmacopoeia (EP).

Standards

History

When I joined NIBSC in 1974 my remit was to establish a laboratory for testing clotting factor concentrates and other coagulation-related products such as heparin, as well as to organize the development of national and international standards for these products. Initially, I worked in the Division of Hormones and Blood Products under Dr Derek Bangham, but a few years later Blood Products became a separate Division with Dr Duncan Thomas as Head. The procedure for establishment of international standards had been in place for many years under Dr Bangham as Head of the Biological Standards Division at the National Institute of Medical Research (NIMR), Mill Hill, before NIBSC was formed in the early 1970s. In fact this procedure started as far back as the 1920s when the NIMR was first formed – curiously enough this was initially in the same building at Hampstead where NIBSC was established, so it could be said that the Standards work eventually came back home. Henry Dale (later Sir Henry) was made Head of NIMR, and was responsible for the first International Standard (IS) for a biological substance, that for insulin. This was followed during the next 15 years by Standards for several other hormones and for antibiotics and antitoxins. Internationally the work was organized initially under the auspices of the League of Nations, via an *ad hoc* Committee, but the main protagonists were NIMR and the State Serum Institute in Copenhagen. In 1947 the newly established World Health Organisation (WHO) established an Expert Committee on Biological Standardisation, which took over all responsibilities in this area. Further details of the history of biological standardization are given in a book by Derek Bangham [11].

Standards in coagulation

The first Standard in the area of haemostasis and thrombosis was the IS for heparin, established in 1942 [12]. In the 1960s work commenced on establishing Reference Preparations for thromboplastin reagents, because of their widespread use in control of oral anticoagulation; these would eventually be established in the 1970s by WHO as International Reference Preparations [13]. In the meantime, work had also begun towards preparations of an IS for one of the clotting factors, FVIII.

First International Standard for FVIII

The need for a standard for FVIII was increasingly recognized during the 1960s as treatment with, first cryoprecipitate, then intermediate purity concentrates started to take hold – it became particularly important when concentrates were manufactured and sold commercially around the world, and were priced by the unit. Although cryoprecipitate was widely used as therapy in the 1960s it was considered unsuitable as a standard because of possible difficulties in freeze drying and uncertain stability. The two materials studied were a normal plasma pool, supplied by the Oxford Transfusion Centre, and an intermediate purity concentrate, supplied by Dr Alan Johnson of New York.

These two materials were ampouled at NIMR and sent out to 20 expert laboratories around the world; each laboratory assayed these samples against their own local standard, which was usually a plasma pool from local donors (i.e. laboratory staff), though in one case was a plasma sample from a single individual, the clinical haematologist himself!

This was the first time that FVIII assays in different labs had been compared on the same samples, and the results were a surprise to many experts in the field. The potency of the concentrate estimated by the various labs covered a 10-fold range! The variability on the plasma sample was considerably less, though still high. This is a graphic illustration of the importance of 'like vs. like', and it might be thought that the high variability in assays of the concentrate would mitigate against its use as a standard. However, bearing in mind the increasing use and availability of FVIII concentrates, it was thought preferable to establish a concentrate standard, on the basis of 'like vs. like'; the concentrate was also more stable than the plasma.

The concentrate was duly established as the first IS for FVIII by WHO in 1970 [14].

In retrospect, there were three reasons for the high variability in assays of the concentrate standard: the number of donors in the local plasma pools was not high enough to take account of the wide normal range of FVIII in the normal population (50–200%); the concentrate was very impure, and non-FVIII components could affect the assays non-specifically – later it was realized that this 'matrix effect' could be minimized by predilution in FVIII-deficient plasma; there was a discrepancy between the one-stage and two-stage method when assaying concentrates against plasma standards – this was masked by the extreme variability within each method and would only be discovered some years later.

Second International Standard for FVIII

In 1974 tests for hepatitis B surface antigen were introduced, and the material in the first IS was found to be contaminated. Although there were still considerable stocks it was deemed necessary to replace it as soon as possible. This coincided with my arrival at NIBSC, so one of the first jobs I was given was to organize the replacement of the FVIII IS. Fortunately, my predecessor, Dr Milica Brozovic, had foreseen the need for a replacement and had ampouled a preparation of an intermediate purity concentrate, obtained from the Lister Institute, Elstree, UK (later the Blood Products Laboratory). My knowledge of standardization procedures at this time was virtually zero, but I was able to call on the long experience of Dr Bangham, and to make use of the international contacts with NIBSC which had been fostered during the work on the first Standard. Following a successful international collaborative study, the second IS was duly established by WHO in 1978 [15]. The second Standard was calibrated against the first, and as both materials were similar in composition this was a true 'like vs. like' comparison; because of this the agreement on potency among participating laboratories was much better than when the first Standard had been calibrated against local plasma pools.

Another great help during the organization of this and other collaborative studies was the support of a strong Statistics Department at NIBSC. One statistician in particular, Tom Kirkwood, had a strong intellectual curiosity about haemostasis assays and he and I developed a good working relationship which led to the publication of several papers.

British Standards for FVIII

The freeze-dried normal plasma which had been ampouled for the study of the first IS had been issued to labs in the UK as a British Standard, and had proved useful for assays of patients' samples and cryoprecipitate; so much so that it had to be replaced at frequent intervals, and my second task was to organize the replacement of the fourth British Standard with the fifth. I had already arranged to visit the MRC Research Unit on Blood Coagulation at Oxford, which at that time was the fount of all knowledge of coagulation in the UK, to get advice on setting up the two-stage assays in my lab, so I took advantage of the opportunity to discuss with Dr Rosemary Biggs about the British Standards. Dr Biggs made a number of pertinent criticisms, notably that the FVIII content of the Standards was too low to be considered normal, that the reports of the collaborative studies, written by a statistician, were 'incomprehensible', and that insufficient quantities were available.

I instituted a number of changes to deal with these criticisms, in particular to avoid losses of FVIII I shortened the time between blood collection and freeze drying (this involved transport of the plasma in my own vehicle). Tom Kirkwood and I tried to write the reports so that they could be understood by laboratory scientists without specialized statistical knowledge, and I distributed the standards in larger amounts, particularly to smaller labs to use as a working standard. The increased usage meant frequent replacements, almost on an annual basis, but the wider availability of the Standards was much appreciated, and hopefully contributed to improved agreement on FVIII assays between laboratories in the UK.

The other main function of NIBSC apart from making national and International Standards was to act as the National Control Laboratory for testing Biological Products, and I soon found increasing numbers of batches of FVIII concentrates coming in for testing (the Therapeutic Substances Act of 1968 had laid down mandatory testing of each batch of biological products by NIBSC before it could be marketed). The numbers increased further when samples started to arrive from the national fractionation laboratories at Elstree and Edinburgh (they had previously been exempt from testing), and it became impractical to use the IS directly to assay all these products. We needed a working standard, and I contacted the Fractionation Laboratories at Elstree and Edinburgh to see if they would support the creation of a British Working Standard for FVIII concentrate, to be shared among all three laboratories. The two centres took up this idea enthusiastically, and it became a long-standing and mutually satisfactory arrangement, whereby one of the two manufacturers would supply the concentrate, and NIBSC would arrange the ampouling and calibration by collaborative study.

Plasma and concentrate standards

In keeping with general practice in standardization, once the first IS for FVIII had been established,

successive batches of British Standards (both plasma and concentrates) were calibrated against it. The assays of the plasma standards were much more variable among labs than those of the concentrates, and when analysing a series of studies, Tom Kirkwood and I noticed that there was a significant discrepancy between the results from one-stage and two-stage assays; the one-stage method gave higher potencies than the two-stage method, by 20% on average [16].

This is another example of the 'like vs. like' principle and it became clear that a separate International Plasma Standard for FVIII would be desirable to calibrate local and commercial plasma standards. Changes in the method of collection and handling of plasma, and in freeze drying techniques, led to improved stability of FVIII in lyophilized plasma, and eventually the first IS for FVIII, plasma was established in 1981 [17], by assay against normal plasma pools in participants' laboratories; it was calibrated also for FVIII:Ag (previously named FVIII C:Ag), and for von Willebrand factor antigen and activity.

Factor IX

As for FVIII, a concentrate standard was the first to be established by WHO, for assay of therapeutic materials; this consisted of a three-factor prothrombin complex concentrate (PCC) [18]. This was established in 1975 and did not need replacement for another 10 years. By this time, experience with FVIII had led to the recognition of the need for an international plasma standard for FIX, as well as a concentrate standard. In the collaborative study, therefore, both a replacement PCC and a plasma standard were calibrated; the latter was also assayed for the other vitamin K dependent factors II, VII and X, and both standards were established by WHO in 1987 [19]. Subsequently high-purity single FIX concentrates were developed, but assays of these against the PCC Standards did not cause any problems. The WHO third IS was a single FIX concentrate as is the current WHO fourth IS. These have been shared among WHO, FDA and the EP, thus avoiding the need for calibration of separate working standards and thereby harmonizing the labelling of FIX concentrates on a worldwide basis.

Standardization of high-purity and recombinant FVIII concentrates

During the 1980s and 1990s continuing developments of plasma-derived concentrates, due to requirements of viral inactivation and improved purification methods, as well as the introduction of recombinant products, considerably broadened the range of FVIII products available. This made the choice of material for the IS important, as it was shown that some concentrates give discrepancies between one-stage and chromogenic or two-stage methods [20].

Early attempts to measure FVIII:C in full-length recombinant FVIII concentrates relative to the WHO third and fourth IS FVIII concentrate (plasma derived), were associated with extremely large inter-laboratory variability, with geometric coefficients of variation (GCVs) ranging from 39 to 137% depending on method [21,22]. Initially, it was considered that a separate IS recombinant FVIII concentrate might be necessary to improve agreement between laboratories. However, subsequent studies revealed that the high variability could be overcome by the following specifications of assay methodology:-

1. *FVIII-deficient plasma*. The use of haemophilic plasma, or deficient plasma with a normal VWF level was found to be essential to give full potency in one-stage assays.
2. *Assay buffers*. It was found that albumin at a concentration of 1% w/v (10 mg mL^{-1}) was necessary in all assay buffers to obtain reproducible results.
3. *Predilution*. Predilution of both test and standard with haemophilic plasma, or its equivalent, was necessary for assay of all recombinant and high-purity plasma-derived products, whichever assay method was used.

These specifications precluded the need for a dedicated recombinant standard and were published as recommendations by ISTH/SSC [23] and also incorporated into the EP monograph for the assay of FVIII [24].

The fifth IS was a high-purity plasma-derived material, but the sixth IS was composed of recombinant FVIII, in recognition of the widespread use of recombinant products, and in anticipation that plasma-derived concentrates would suffer a rapid decline in production and use. However, in the event most manufacturers have continued to produce plasma-derived products, and as there are still more plasma-derived products than recombinant ones, the seventh IS and current eighth IS reverted to plasma-derived products.

In the calibration of these standards there has been good agreement between laboratories; discrepancies between one-stage and chromogenic or two-stage methods were less than 10% for the sixth and seventh IS [25,26] and there was absolute agreement for the eighth IS. However, this is not always the case – several concentrates have shown larger discrepancies when assayed against the IS, including the EP standard and the Mega 2 Standard [27]; in the latter case the difference between one-stage and chromogenic potencies was over 30% and it was decided to label this standard with a different potency for each method. The B-domain deleted recombinant concentrate also has a large discrepancy between methods, and even between different types of chromogenic or one-stage method [28]. These differences appear to be an inherent

property of the materials and as far as possible it is best not to use such materials for standards – fortunately the majority of FVIII concentrates do not give discrepancies between methods when assayed against the IS using the ISTH/SSC recommendations.

Plasma and concentrate units and *in vivo* recovery

The assay of FVIII concentrates against plasma standards has been a longstanding problem because of wide variability among laboratories and a basic difference between assay methods, and for this reason two separate WHO standards for plasma and concentrates were developed. However, although such comparisons are avoided in routine assays, they are relevant to manufacturers of plasma-derived concentrates, and especially to clinicians measuring *in vivo* recovery. In the latter situation, patients' post infusion samples, which essentially consist of concentrates 'diluted' in the patient's haemophilic plasma, are usually assayed against a plasma standard.

As already noted it was first found in 1978 [16] that when concentrates were assayed against plasma the potencies were higher by the two-stage method than by one-stage assays – the average discrepancy from a number of collaborative studies at this time was 20%. Since then the same trend has been found in almost every collaborative study, although the size of the discrepancy varies from study to study, and possibly with different types of concentrates.

In recent years, the chromogenic method has largely replaced the two-stage clotting method for assay of concentrates, and not surprisingly it also gives higher results than the one-stage method, being based on the same principles as the two-stage. Despite considerable investigation the basic causes of this discrepancy remain unknown, although it is thought that the extensive processing applied to both plasma-derived and recombinant concentrates could lead to differences in their rates of activation and inactivation in the two method types from the FVIII in normal plasma, and there is some evidence for this from recent studies [29].

A resolution of this problem is only possible when the exact causes of the discrepancy are discovered; it may then be possible to adjust one or both of the methods to give similar values. In the meantime, a practical solution which has been discussed by the FVIII/FIX Subcommittee of ISTH/SSC is to regard the post infusion samples as concentrates, 'diluted' in a patient's plasma, which is essentially what they are, and use a concentrate standard, diluted in haemophilic plasma, instead of a plasma standard, to construct the standard curve. However, the nature of the concentrate standard needs to be carefully considered; it should be as similar as possible to the injected product.

This approach has been tested in a number of *in vivo* recovery studies, and the discrepancy between one-stage and chromogenic methods using the plasma standard was completely abolished with the appropriate concentrate standard [30]. However, in one case the use of a concentrate standard, in this case not the same as the product infused, made the situation worse. Therefore, the use of concentrate standards needs to be product specific, and should probably be restricted to recombinant and very high-purity plasma-derived products.

Modified products

Most recently, a number of modified FVIII and FIX concentrates have been developed with novel properties, introduced through structural or chemical modifications (e.g. truncation, pegylation, fusion) to improve manufacturing yield or to prolong plasma half-life. These will challenge the traditional approach to potency labelling relative to the WHO IS [31,32].

Potency estimation of pegylated versions of both FVIII and FIX, by the one-stage clotting method, appears to be associated with particular issues relating to the direct interference of the polyethylene glycol with some APTT reagents [33], and it may be necessary in some cases to use product specific standards for monitoring.

However, there are indications that most modified products are amenable to potency estimation using conventional methods. Nonetheless, decisions on the potency labelling should be guided by a thorough characterization, *in vitro* relative to the WHO IS, which should include the effect of different reagents (e.g. APTT reagent) and where method discrepancies exist there will need to be agreement between licensing authorities and manufacturers on the appropriate method for potency labelling of individual products. Recommendations for the assay of modified FVIII and FIX products have been developed by ISTH/SSC and recently published [34].

Conclusions

Looking back over the last 50 years, considerable progress has been made in the standardization of laboratory tests and assays. Compared with the 10-fold range of potencies found for the FVIII concentrate in the first international collaborative study, in recent studies all labs are within 20% of the mean. It is also gratifying that all manufacturers, both of concentrates and of diagnostic plasma standards use International Standards to calibrate their products. The new products present challenges to standardization, but with the spirit of cooperation, which has developed over the years between manufacturers, regulatory agencies and clinical labs these can be overcome.

Disclosures

The author stated that he had no interests which might be perceived as posing a conflict or bias.

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