THE ASSAY OF ANTI-HAEMOPHILIC GLOBULIN

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The Biggs, Eveling and Richards assay of anti-haemophilic globulin is examined and some modifications discussed. The statistical design and analysis is offered in a simple form suitable for routine application. The relationships of some defects in the assay to the property of the phospholipid are discussed. It is shown that the method is satisfactory if certain precautions are taken, and if the interpretation is carried out objectively by mathematical methods. Serious errors can arise from neglect of these precautions. Modified assays are described, one of which could probably be used in cases of serious difficulty with reagents. The other apparently depended on the properties of a particular batch of prothrombin. The method was abandoned and is reported here because it is the only modification that gave promise of being more accurate than the original. It is emphasised that the assay is based on hypotheses that have been satisfactorily verified, but that are well recognised to be incomplete. The course of the reaction is by no means clear, and it is unlikely that the assay can accurately be used to compare materials widely dissimilar in qualitative properties.

In the original assay of anti-haemophilic globulin (A.H.G.) published by Biggs, Eveling and Richards[1] a system of reagents is used consisting of isolated but impure blood coagulation factors. From these factors thromboplastin is generated in a quantitative manner related to the quantity of A.H.G. in the system. It can therefore be used for the assay of A.H.G. by measuring the amount of thromboplastin as indicated by the clotting time of a recalcified plasma substrate.

In the method first published the assay was interpreted by comparing the clotting time of the unknown material with a standard curve based on a long series of measurements of another batch of material used as a standard. When we began to use this method in 1954, we found that the relationships between the clotting time and concentrations of A.H.G. were expressible by the commonly observed linear correlation of the logarithm of the dose and the logarithm of the effect, and this has now become standard practice[2].

It is the purpose of this paper to describe the detailed modifications in technique and analysis that we have made to the original method. Some of the difficulties which are now arising in its application are not due to defects in the method, which is a good example of a bioassay, but to the fact that the use of statistical methods of analysis is not common enough in haematology. One result of this is that unsuitable experimental designs are sometimes used, and the magnitude of the errors may be underestimated or overestimated but is rarely measured. We can also contribute some observations on the necessary properties of the reagents used in the assay, although much remains obscure in this complicated field.

The Assay

It is unnecessary to describe the assay process in detail, since the existing accounts are adequate; but to make the present paper comprehensible the following is a brief description.

A system is set up in which Factor V, serum containing Factor VII and Christmas Factor, a phospholipid prepared from human brain, A.H.G. and calcium chloride are allowed to react for 10 minutes. The thromboplastin formed is related to the concentration of A.H.G. and is assayed by adding portions of the incubation mixture to a substrate, consisting of human citrated or oxalated plasma, simultaneously re-calcified, and observing the clotting time. A standard preparation is treated in the same way at approximately the same time, and the results are interpreted by comparing the clotting times in the manner described below.

The Technique of the Test

All reagents including A.H.G. dilutions are stored in the ice bath throughout the assay, since in the course of our investigations we sometimes found that significant changes took place when reagents were kept at room temperature for some hours.

The standard and unknown A.H.G.'s to be assayed are wetted at the same time and when dissolved made up to 1 in 10 with saline. One in 100 dilutions are made from all these and kept on ice.

For a 6-point analysis, doubling dilutions from 1 in 400 to 1 in 3,200 of standard are prepared (the three best consecutive points being chosen for the final mathematical analysis). For a 4-point analysis quadrupling dilutions are in general the best, and in this case 1 in 400 and 1 in 1,600 are appropriate. These dilutions are prepared with a 1 ml. bulb pipette, adding saline from a 10 ml. graduated pipette.

All tubes are mixed by inversion 6 times. When diluting the next sample to be assayed, a new standard must be diluted at the same time.

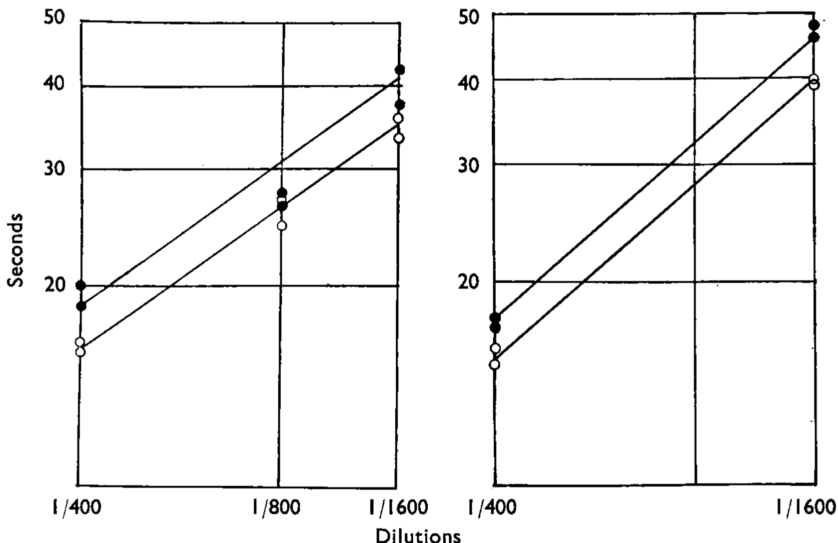
Using Pasteur pipettes graduated at 0·1 ml., incubation mixtures consisting of 0·1 ml. phospholipid, 0·1 ml. Factor V and 0·1 ml. serum, are made up and 0·1 ml. of each A.H.G. dilution (discarding the first 0·1 ml. taken up in the pipette and starting at the weakest dilution) is added to the 8 tubes in order. It is observed that if more than 8 tubes are used in one test the last ones may be erratic, and in the 6-point assay, where we actually test duplicates of 4 dilutions and discard one, we divide the assay into two halves, each with one set of the duplicates. Statistical analysis by the methods of Bliss and Marks[3] showed that the error due to lapse of time between the two halves of the assay was not significant unless the time was long. The order of the tubes is then randomised in the rack by one of the well-known methods. A convenient practical scheme is to write the numbers "S1", "S2" and so on, on the ends of corks, keep these in the pocket and take out one at a time to decide the order.

0·1 ml. $CaCl_2$ solution is added at 1 minute intervals to the tubes in their random order. As soon as the calcium chloride is added to a tube it is shaken and replaced in the water bath at 37°.

A clotting tube containing 0·1 ml. of plasma is put in the water bath at about the ninth minute. 0·1 ml. of the incubation mixture from the first tube is pipetted and discarded. Then 0·1 ml. is taken and added to the plasma tube simultaneously with 0·1 ml. $CaCl_2$ solution starting the stopwatch at the same time. The tube is taken out of the water bath, dried quickly and held in a suitable position, revolving it backwards and forward until a web is perceived. The watch is then stopped and the time entered serially in the note-book, previously prepared to agree with the particular random order in use. The process is repeated on the remaining tubes at one minute intervals.

Statistical Analysis

A simple method of plotting on double logarithmic paper is illustrated in Figures 1 and 2. For rapid use in a clinical laboratory, this may be all that is necessary and, in fact, a *good* assay analysed graphically, rarely



FIGS. 1 and 2. Illustrations of 6 point and 4 point Assay. The points are plotted directly from the experimental results and a pair of parallel lines drawn in by eye to lie evenly among the points. The ratio of the potencies of the two samples, assumed to be present in equal concentrations by weight, is given by the ratio of the readings on the dilution scale corresponding to any point on the time scale.

provides a result significantly different from that given by mathematical methods. One of the inherent difficulties of this assay, particularly when it is being used to compare materials both containing A.H.G. but which are not otherwise identical (for example, normal human plasma and animal A.H.G.) is that the lines drawn may not be parallel. As is well known, this indicates a qualitative difference between the materials and it must be stressed that the advice of the statisticians cannot safely be ignored—they have provided tests of validity which eliminate assays in

Scheme for duplicated 4-point Analysis (Gaddum. *Quadrupling dilutions.* Batch No. *206C.* Date: *7.6.56.*

	U1	U2	S1	S2		
	2041	*5997*	*2504*	*6628*		
	1818	*5925*	*2355*	*6776*		
Sum	*3859*	*11922*	*4859*	*13404*	Sum of	
Diff.	*223*	*72*	*149*	*148*	Diffs.	
Mean	*1929*	*5966*	*2429*	*6702*	*592*	= Q

$$\frac{Q}{4{\cdot}52} = \frac{592}{4{\cdot}52} = 130 = S$$

$$\frac{S^2}{2} = \frac{16900}{2} = 8450 = \text{V or A}$$

$$\frac{(U1 + U2-(S1 + S2)}{2} = \frac{7895-9131}{2} = -618 = F$$

$$\frac{(U2 + S2-(U1 + S1)}{2} = \frac{12668-4358}{2} = 4155 = E$$

$$(U2 + S1)-(U1 + S2) = 8395-8631 = -236 = G$$

$$= \frac{E}{-0{\cdot}6020} = \frac{4155}{-0{\cdot}602} = -6900 = b$$

$$\frac{F}{b} = \frac{-618}{-6900} = 0{\cdot}0895 = M$$

Antilog M = potency ratio = *1·228* = R

Validity Tests

Parallelism $t = \frac{G}{2\sqrt{V}} = \frac{-236}{184} = 1{\cdot}28$ $\quad t = 2{\cdot}78$

$$V(b) = \frac{V}{0{\cdot}36} = \frac{8450}{0{\cdot}36} = 23400 = V(b)$$

$$g = \frac{V(b) \times 7{\cdot}72}{b^2} = \frac{23400 \times 7{\cdot}72}{47500000} < 0{\cdot}1 \quad g$$

Fiducial Limits. Use appropriate alternative according to value of g.

$g > 0{\cdot}1$

$$2 + \frac{gM}{1-g} \pm \frac{2{\cdot}78}{b(1-g)} \sqrt{A(1-g) + V(b)M^2}$$

$g < 0{\cdot}1$

$$2 \pm \frac{2{\cdot}78}{b} \sqrt{A + V(b)M^2}$$

Log Limits = *2 ± 0·035* = *1·965–2·035*

Limits = *92–108* per cent

FIGS. 3A and 3B. The simplification results from a process of standardisation of procedure, which allows variables to be treated as constants. The omission of the characteristic of the logarithms is possible because in practice all the times in seconds have two digits.

The entry "$t = 2{\cdot}45$" is only a reminder that the calculated value in the validity tests must not exceed this number.

Values of g greater than 0·1 rarely occur. When they do, the assay is generally useless and has to be repeated. The left-hand formula is, therefore, hardly ever used but can sometimes give limited information in the case of urgency. Its value is to reveal the extent of the unreliability of unsatisfactory assays. Having arrived at the potency ratio R the activity of the unknown is simply found by multiplying the activity of the standard by this number, making due allowance for dilutions.

Scheme for duplicated 6-point Analysis (Gaddum). *Doubling dilutions.* Batch No. 59.
Convert all times to logs, and omit characteristic. Date 9.2.58

	U1	U2	U3	S1	S2	S3	
	3010	*4346*	*6274*	*2148*	*4314*	*5490*	
	2695	*4216*	*5752*	*1987*	*3945*	*5263*	
Sum	*5705*	*8562*	*12026*	*4135*	*8259*	*10753*	Sum of
Diff.	*315*	*130*	*522*	*161*	*369*	*227*	Diffs.
Mean	*2852*	*4281*	*6013*	*2067*	*4129*	*5376*	*1724* = Q

$\frac{Q}{6{\cdot}78} = \frac{1724}{6{\cdot}78} = 254$ = S

$\frac{S_2}{2} = \frac{64500}{2} = 32250$ = V

$\frac{(U1 + U2 + U3)-(S1 + S2 + S3)}{3} = \frac{13146-11572}{3} = 524$ = F

$\frac{(U3 + S3)-(U1 + S1)}{4} = \frac{11389-4919}{4} = 1617$ = E

$\frac{(U3 + S1)-(U1 + S3)}{2} = \frac{8080-8228}{2} = -74$ = G

$U3 + U1-2\,(U2) = 8865-8562 = 303$ = H_1

$S3 + S1-2\,(S2) = 7443-8258 = -815$ = H_2

$-\frac{E}{0{\cdot}3010} = -\frac{1617}{0{\cdot}3010}$ — 5370 = b

$\frac{F}{b} = -\frac{524}{5370} = -0{\cdot}0975$ = M

Antilog M = potency ratio = *0·7989* = R

Validity tests $t = 2{\cdot}45$

Parallelism $\frac{G}{\sqrt{V}} = \frac{74}{180} = 0{\cdot}412$

Curvatures $\frac{H_1}{\sqrt{6v}} = \frac{303}{441} = 0{\cdot}688$

$\frac{H_2}{\sqrt{6v}} = \frac{816}{441} = 1{\cdot}85$

= A

$A = \frac{2V}{3} = 21500$ $V(b) = \frac{V}{0{\cdot}364} = 89000$ = V (b)

$\frac{V(b)\,t^2}{b^2} = \frac{89 \times 6}{29000} = \frac{535}{29000} = <0{\cdot}1$ = g

Fiducial limits. Use appropriate alternative according to value of g.

$g>0{\cdot}1$

$$2 = \frac{gM}{1-g} \pm \frac{2{\cdot}45}{b\,(1-g)} \sqrt{A\,(1-g) + V\,(b)\,M^2}$$

$g<0{\cdot}1$

$$2 \pm \frac{2{\cdot}45}{b} \sqrt{A + V\,(b)\,M^2}$$

Log Limits = 2 ± *0·0680* = *1·9320–2·0680*
Limits = *86–117* per cent

which the fundamental requirement of similarity of response is objectively tested.

Since a proposed standard for A.H.G. is the activity in 1 ml. of normal pooled human plasma, difficulties over validity and relevance may arise. In our opinion there is need for a standard consisting of a certain quantity of dry A.H.G. of agreed activity. It is true that as with other biological standards, there is no guarantee that this will remain stable for prolonged periods. The ultimate standard may therefore well be the average level of A.H.G. in the population at large, which is unlikely to change, but a comparison at rare intervals to establish that the dry standard has not significantly changed is a very different thing from the regular use of a standard material which is qualitatively different from the drug itself. Material called A.H.G. is without doubt extremely complex and plasma far more so. Unless the material under test is as like as possible to the standard, trouble is almost sure to arise.

Another "disease" of this assay is a poor slope. Since slope and random error enter into the calculation of limits as their squares, the combination of poor slope and not very good agreement between duplicates can make an assay almost useless. This is not easy to detect without mathematical analysis. The B.P. provides suitable methods of analysis based upon the paper by Gaddum[4]. Both Gaddum and the B.P. have rightly provided for a variety of schemes to cover many requirements, but in doing so have left the potential user with a choice I feel he would often prefer not to make. In Figures 3*a* and 3*b* are given therefore the only two schemes we have found it necessary to use, so simplified that technicians work out their own results as a matter of routine. If these forms were accepted as the right way of working out an A.H.G. assay without worrying about the reasons, a considerable advance would be made towards objectivity.

Reagents

All the organic reagents used in this assay, including the A.H.G., are complex and imperfectly purified native clotting factors. The system is not far removed from life, and it is a tribute to the perspicacity of its authors that they have reproduced in a test tube much of what happens in the body. It is, therefore, no surprise to find that the properties of the reagents vary. The serum (providing Christmas factor and factor VII) and factor V rarely give difficulties once the proper concentration and incubation time for any particular batch have been found. The trouble is almost always due to the phospholipid, and it is possible that preparations are in use that would not give reasonably accurate results if mathematical analysis were used to measure the errors.

The original work at Oxford and by ourselves was carried out on one particular batch of phospholipid. When this preparation was repeated, both at Oxford and in these laboratories, it was found that the necessary properties were not always present. The slope was often too poor to be useful because the times at high concentrations of A.H.G. were too long, and those at low concentrations too short. Acting on the hypothesis that there were two components in the phospholipid, one an accelerator and

the other an inhibitor, we prepared numerous varieties of phospholipid from the brains of cattle, pigs, horses, man and also from soya beans, and fractionated some of them by various methods. In one such attempt we prepared an inhibitor from human brain by the method of Rapaport, Aas, and Owren[5]. By using a soya bean phospholipid[6] in the incubation mixture and this inhibitor in the substrate, we produced a system which although not as good as the brain phospholipid, gave reasonably accurate results in trial. We then fractionated crude brain phospholipids and confirmed the observations of Newlands[7] to the extent that some of these fractions are inhibitory and some are accelerators, as we had imagined. The position is made more complicated by the fact that a phospholipid may be an accelerator at one concentration but an inhibitor at another. We had limited success by combining these purified fractions in the assay. We have also found that two crude preparations, one of which gives a short time but a poor slope, and the other gives long times, could give good results by being mixed in suitable proportions (Fig. 4). The mixture is less stable than a suitable crude preparation. We have observed that a deterioration in slope of a mixture can be corrected by adding more of the material giving long times.

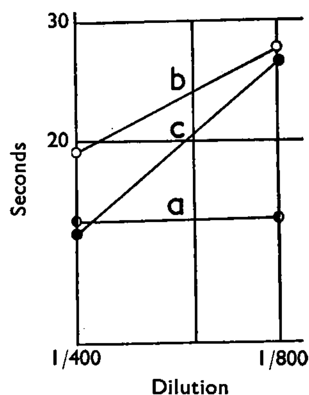


FIG. 4. The difference in properties of phospholipids in this system:
(*a*) is a preparation of high activity in the generation of thromboplastin, but quite useless in the assay for it is not sensitive to varying concentrations of A.H.G,
(*b*) of improved sensitivity, but with rather poor activity,
(*c*) is a mixture of the two and shows high sensitivity and good times.

When these observations are compared with the repeatedly observed fact that deterioration of a phospholipid suspension almost always shows itself in a deterioration of slope, it seems possible that we are indeed witnessing the destruction, probably by oxidation, of an inhibitor less stable than an accelerator but both essential for the assay.

There is, however, an alternative hypothesis. We found during the investigations on soya bean phospholipids that a fraction having no thromboplastic activity, when tested by the one stage prothrombin test, developed activity when incubated in the usual mixture with serum, factor V and calcium chloride, but without A.H.G. The activity was of the same order as that of A.H.G. and a logarithmic relation between concentration of phospholipid and clotting time was found. It is, therefore possible that certain specimens of phospholipids may be active in this system, even in the absence of A.H.G. and could account for unsatisfactory slopes for, in effect, a constant quantity of activity would be added at all dilutions thus reducing the supposed ratios between the dilutions.

We believe that we now have a limited degree of control over the properties of the phospholipid component of the incubation mixture, but

much further work is required before it will be possible to say that all the difficulties have been removed, if, indeed, this ever happens. At this point we must emphasise that preparations that are highly active as shown by very short clotting times may be quite useless in the assay, which requires, not short times, but a high sensitivity to the variations in A.H.G. concentrations. Figures 1, 2 and 4 show a selection of some of our experimental results illustrating the foregoing descriptions.

The Substrate

After trying numerous combinations of the reagents prepared from various animals with various substrates, we concluded that the only suitable substrate was fresh human plasma. Bovine plasma has been tried but in our hands this has always given poor slopes, and the experience at Oxford was similar. We once succeeded in devising a system somewhat resembling that of Wolf[8] in which bovine prothrombin was added to the incubation mixture so that thrombin was produced there. Excellent results were obtained, more accurate than the original method, because of the greatly improved slope and capable of being tested on a bovine substrate. When this batch of prothrombin was used up, we attempted to reproduce it with quite unsatisfactory results and we had to abandon this line of enquiry.

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After the Author presented the paper there was a DISCUSSION. The following point was made.

The state of purity of the phospholipid might account for the difficulties encountered with this substance. A limit to the lysophosphatide fraction might be desirable. Hens' eggs were suggested as the most reliable source of phosphatide.