Assay of Plasma Clotting Factors Using Parallel-Line Bioassay Principles

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(Sysmex J Int 13 : 37-42, 2003)

Key Words Plasma Clotting Factors, Assay Validation

Received 24 December, 2003; Accepted 7 January, 2003

INTRODUCTION

The specific quantification of clotting factors is central to the laboratory investigation of a prospective haemorrhagic diathesis or prothrombotic state. Plasma clotting factors are assayed by assessing the degree to which dilutions of a test and reference plasma correct the clotting time of a substrate plasma (specifically deficient in the factor) measured in a suitable test system. The test system used is dependant on the clotting factor investigated. The prothrombin time (PT) system is commonly used for the assay of factors II, V, VII, X and the activated partial thromboplastin time (APTT) for factors VIII, IX, XI, XII.

The range of sample dilutions to be used in an assay system are determined by testing a wide range of normal plasma dilutions (e.g. doubling dilutions 1/2 – 1/1024). This process produces a classical sigmoid dose response curve with a central linear component (***Fig. 1***). The sample dilutions to be used are selected to produce a series of clotting times which fall towards the middle of that central linear component. Data produced in an assay of this type can be analysed by graphical means[1], comparing the dose response of serially diluted test plasma with corresponding dilutions of a standard (or reference preparation). Plotting the clotting times against log concentration in this procedure allows not only quantification, but

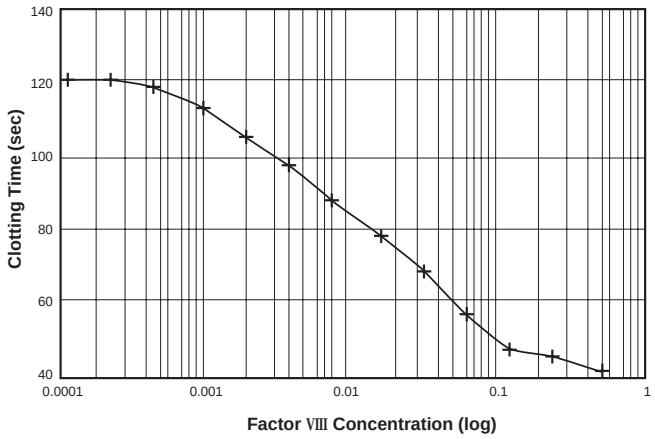


Fig. 1 *Factor VIII assay data illustrating a sigmoid dose response over a wide range of dilutions*

also reveals evidence of inhibition or sample activation, processes which can either be of pathological or artefactual origin.
In the absence of an inhibitor or sample activation, the relationship between the logarithm of the dose (i.e. sample dilution) and the response (i.e. clotting time) is represented by a straight line over the range of dilutions tested. For any unknown preparation the straight line is parallel to that for the standard. Using these procedures the experienced scientist can make a visual assessment of the relationship (linearity and parallelism) between the reference preparation and the sample under test. However, data analysed solely by graphical means introduces subjectivity associated with fitting lines to data points visually and ignoring the information which optimal experimental design together with subsequent statistical analysis would yield[2-5]. To obtain a more complete analysis of coagulation assay data a number of computer programs have been described[6-8], but these descriptions supply only general principles of bioassay programs[6] or are for use on large computer systems[7], and are not readily introduced into clinical haemostasis laboratories.

LABORATORY AUTOMATION

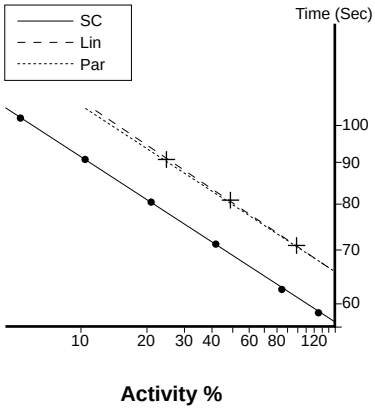
Most automated coagulometers have the ability to perform clotting factor assays. A commonly adopted procedure is to process a standard (calibration) curve for a specific clotting factor and subsequently test a single dilution of a test plasma. Sample relative potencies are then derived from the stored standard curve. Such analyses are flawed because (i) reference preparations and test samples must be processed at the same time to reduce the effect of temporal drift, (ii) multiple dilutions of both reference preparation and test samples must be analysed to ensure parallel and linear dose response. Bioassays which do not assess these criteria are invalid, with the potential of producing misleading results.

SYSMEX CA SERIES ANALYSERS

The assay procedure adopted on Sysmex CA series analysers employs a utility which they have called MDA (standing for Multi Dilution Assay. ***Fig. 2***). To perform an assay a standard curve of up to six points is processed, samples are tested using the analyser's "sample specific" option which allows the user to select the most appropriate MDA dilution series (***Table 1***). Data from the standard curve and MDA dilution series are presented graphically with least squares lines of best fit constructed for the two preparations (i.e. standard and test plasma). A further line, parallel to the line of regression for the reference preparation, is drawn through the data point for the lowest dilution of the test preparation, bringing any deviation from parallelism to the attention of the instrument operator. To support this function an index to show the extent the MDA line deviates from parallelism with the standard curve, the slope ratio (SR) was added to system software versions 00-16 upward and 00-20 upward on CA-1500 and CA-7000 respectively.
The importance of MDA is illustrated in ***Fig. 3***. ***Fig. 3a*** shows data from a mild haemophiliac with a factor VIII relative potency of 40.0iu/dL, dilutions of the patient's sample exhibit a linear dose response (Test r = –0.999) which are parallel to the standard curve, a condition which fulfils the criteria for assay validation. In contrast a sample from a severe haemophiliac (factor VIII < 1.0iu/dL. ***Fig. 3b***) does not exhibit a dose response (i.e. the clotting times do not get longer as the sample is further diluted) as there is no factor VIII to correct the clotting of the factor VIII deficient substrate plasma. The patient's clotting times are also clearly not parallel to the standard curve, and had data from a sample buffer blank been presented it would have been observed that the clotting times of this and the three dilutions of the patient's sample were essentially the same. In situations like this, it is not possible to state if the patient is simply deficient

VIII MDA

MDA Ratio	Clot time	Activity %
1/1	70.6 sec	44.0 iu/dL
1/2	80.9 sec	41.2 iu/dL
1/4	91.1 sec	42.6 iu/dL
		Mean 42.6 iu/dL
SCr= –1.000	Test r= –0.999	
SR= 1.03		



From the Table above
SCr = Correlation coefficient for the standard curve
Test r = Correlation coefficient for the test sample
SR = Slope ratio

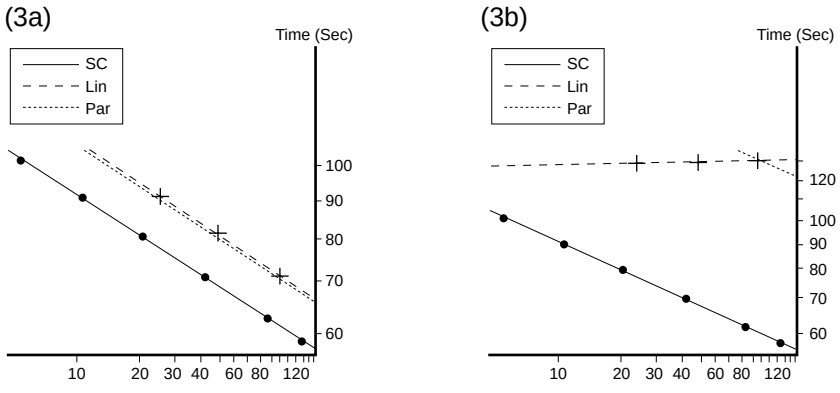
From the Graph above
SC (unbroken line) = Line of regression through the standard curve
Lin (broken line) = Line of regression through the test sample data
Par (dotted line) = Line parallel to the standard curve passing through the lowest dilution of the test sample data

Fig. 2 *Sysmex CA series MDA utility*

in the factor assayed or if they have an antibody to that clotting factor, that can only be elucidated by specifically looking for progressive inhibition in time course studies. Other phenomena which can lead to an invalid assay include sample activation (e.g. by trace amounts of thrombin or activation of the contact sequence. ***Fig. 3c***) and the presence of an inhibitor such as an anti-phospholipid antibody (***Fig. 3d***), in both of these situations the

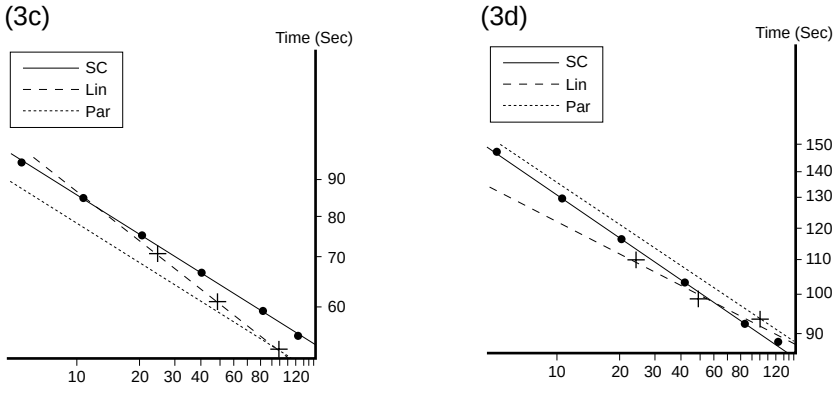
Table 1 *MDA dilution with factor assay*

MDA options	Dilution Sequence	Sample Dilution	Corresponding Activity
Low	2 / 1	1 : 5	200%
	5 / 1	1 : 2	500%
	10 / 1	1 : 1	1000%
Normal	1 / 1	1 : 10	100%
	1 / 2	1 : 20	50%
	1 / 4	1 : 40	25%
High	1 / 8	1 : 80	12.5%
	1 / 16	1 : 160	6.25%
	1 / 32	1 : 320	3.13%



Sample from a mild haemophiliac (40.0iu/dL). The patient's sample exhibits a linear dose response that is parallel to the standard.

Sample from a severe haemophiliac (< 1.0iu/dL). The patient's sample does not exhibit a dose response and is clearly not parallel to the standard.



Sample showing evidence of activation. The patient's dose response is linear but not parallel to the standard.

Sample showing evidence of an inhibitor. The patient's dose response is linear but not parallel to the standard.

Fig. 3 *Factor VIII assays demonstrating the benefit of multi-dilution analysis*

patient's dose response curve may be linear but it is not parallel to the standard curve. Typically, in a normal dose response the difference between the first and second clotting times is the same as the second and third (e.g. If the sequence were: 50 seconds, 60 seconds, 70 seconds, the difference in each case is 10 seconds). However if the sample was activated there is usually a greater difference between the first and second dilutions than the second and third, conversely if there were a lupus anticoagulant (LA) the difference between the first and second dilution would be less than that seen between the second and third dilutions. A practical means of avoiding erroneous results attributed to the presence of LA would be to perform assays with an APTT reagent such as Actin FS (Dade Behring) which is insensitive to lupus anticoagulant[9] (***Fig. 4***).

THE SLOPE RATIO (SR)

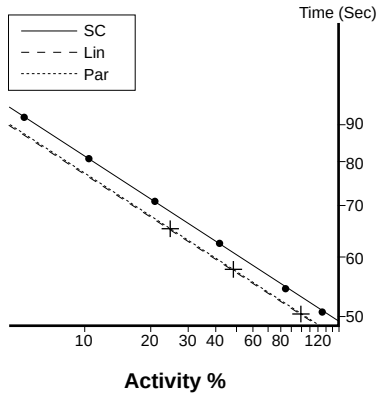
In order to assess the value of SR it was necessary to select samples which would demonstrate gradations of either activation or inhibition. Although sample activation would generate a non-parallel dose response (typically these samples exhibit a greater difference between the first and second dilution than the second and third), to obtain or generate samples with varying degrees of activation would be problematical. Similarly, plasmas containing pathological inhibitors such as lupus anticoagulant or antibodies to specific clotting factors could not be readily tailored to evaluate the slope ratio utility. Therefore various chemical and therapeutic compounds were considered.

We investigated the usefulness of SR by performing one-stage factor VIII clotting assays on a normal plasma spiked with a high affinity, specific inhibitor of human plasma kallikrein. The advantages of using this specific inhibitor were: (i) high specific activity meant there was minimal sample dilution attributable to its addition to plasma (5µL to 1mL of plasma); (ii) the same volume (of various dilutions) of inhibitor were added to the plasma to achieve a range of final concentrations (buffer blank, 1µg/mL, 5µg/mL, 10µg/mL, 20µg/mL). The samples were assayed using the MDA utility, the clotting times from which were also entered into a parallel-line bioassay programme[7] for formal statistical assessment. ***Fig. 5*** show the effect of progressively increasing the concentration of inhibitor. Initially (***Fig. 5a***) the plasma pool prior to spiking with kallikrein inhibitor was assayed, demonstrating (graphically[1] and mathematically[7]) a linear dose response which was parallel to that of the reference plasma. In ***Fig. 5b***, the data is statistically non-parallel ($p <$

(4a)

VIII MDA

MDA Ratio	Clot time	Activity %
1/1	51.0 sec	129.2 iu/dL
1/2	58.5 sec	123.4 iu/dL
1/4	66.0 sec	128.9 iu/dL
		Mean 127.2 iu/dL
SCr= −0.999	Test r= −0.999	
SR= 1.00		



(4b)

VIII MDA

MDA Ratio	Clot time	Activity %
1/1	93.4 sec	81.9 iu/dL
1/2	99.5 sec	111.6 iu/dL
1/4	110.7 sec	117.1 iu/dL
		Mean 103.5 iu/dL
SCr= −0.999	Test r= −0.989	
SR= 0.74		

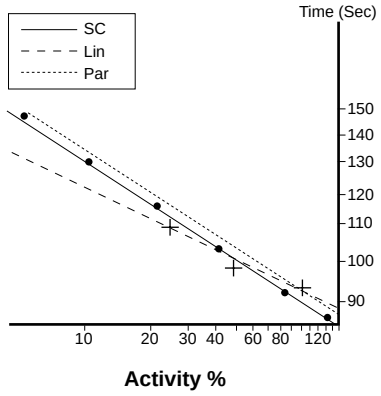


Fig. 4 *Factor VIII assay of a sample containing lupus anticoagulant*
(a) Using a lupus insensitive APTT reagent (Actin FS)
(b) Using a lupus sensitive APTT reagent (Pathromtin SL)

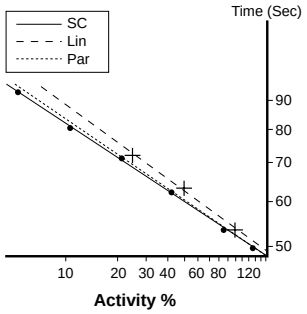
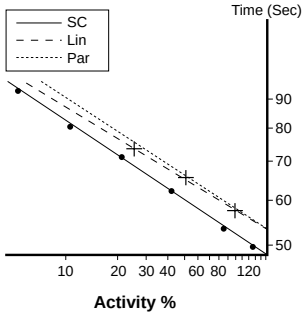
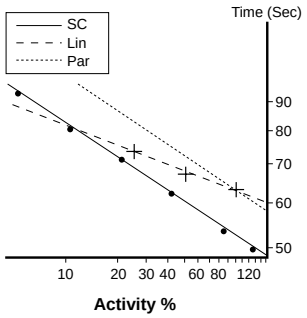
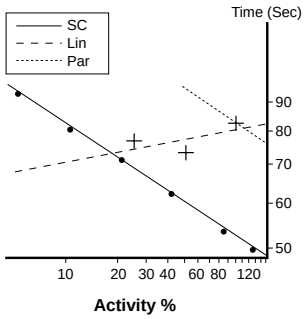
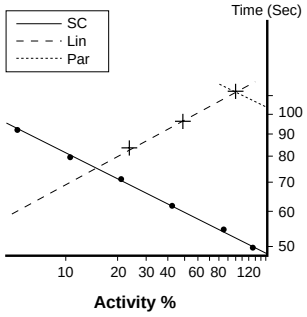
MDA printout from Sysmex CA-7000		Parallel-line-bioassay programme
(5a) 	*kallikrein inhibitor* buffer blank SCr = −1.000 Test r = −0.998 SR = 1.11	**Variance Ratios** Parallelism = 4.1016 * linearity = 0.8474 * ***Not Significant at the 5% level** Relative Potency = 82.8 iu/dL
(5b) 	*kallikrein inhibitor* 1 μg/mL SCr = −1.000 Test r = −0.999 SR = 0.91	**Variance Ratios** Parallelism = 15.7936 ** linearity = 0.1705 * ***Not Significant at the 5% level** ****Significant at the 1% level** **Relative Potency = 68.7 iu/dL** ***Result unreliable***
(5c) 	*kallikrein inhibitor* 5 μg/mL SCr = −1.000 Test r = −0.994 SR = 0.58	**Variance Ratios** Parallelism = 197.4652 ** linearity = 3.8394 * ***Not Significant at the 5% level** ****Significant at the 1% level**
(5d) 	*kallikrein inhibitor* 10 μg/mL SCr = −1.000 Test r = 0.634 SR = −0.28	**Variance Ratios** Parallelism = 3465.4334 ** linearity = 145.3059 ** ****Significant at the 1% level**
(5e) 	*kallikrein inhibitor* 20 μg/mL SCr = −1.000 Test r = 0.998 SR = −1.04	**Variance Ratios** Parallelism = 4461.1377 ** linearity = 7.1949 * ***Significant at the 5% level** ****Significant at the 1% level**

Fig. 5 *Effect of increasing inhibitory action on parallelism*

0.05) but graphically it is unclear whether there is a relevant deviation from parallelism, (although clearly there was a noticeable difference between the relative potencies obtained in ***Figs. 5a and 5b*** [82.8iu/dL and 68.7iu/dL respectively]). ***Figs. 5c – 5e*** exhibit marked non-parallelism and clearly statistical analysis, although it confirms the observation, is unnecessary since the changes are so grossly apparent. Using the information from the multidilution assay utility as criteria for assay validation necessitates that the correlation coefficient for the standard curve (SCr) and correlation coefficient for the test sample (Test r) are both very close to 1.0 (typically between 0.997 and 1.0) to ensure both preparations are exhibiting a linear dose response. To ensure parallelism between the standard curve and test sample the slope ratio (SR) parameter should also be very close to 1.0 (typically between 0.90 and 1.1).

CONCLUSIONS

Each of the MDA parameters are therefore potentially of benefit in the clinical laboratory for flagging assay results which would be equivocal from graphical presentation, or for preventing automatic validation of results that are clearly invalid. This utility offers a compromise between formal statistical analysis and subjective graphical presentation of data. However, it is unfortunate that the manufacturers have given no indication as to what constitutes a statistically significant slope ratio value.

References

1) *Austen DEG, Rhymes IL: A Laboratory Manual of Blood Coagulation, pp.81–89, Blackwell Scientific Publications, Oxford, 1975.*
2) *Ingram GIC: Blood coagulation factor VIII: genetics, physiological control and bioassay. In: Advances in Clinical Chemistry Edited by H. Sobotka & C.P.Stewart, pp.222–235, Academic Press, New York, 1965.*
3) *Ingram GIC, Brozovic M, Slater NGP: Bleeding Disorders: Investigations and Management, pp. 305–325, Blackwell Scientific Publications, Oxford, 1982.*
4) *Finney DJ: Statistical methods in biological assay (2nd Edition). Charles Griffin & Company Ltd., 1978.*
5) *Assays depending upon quantitative response. In: 3rd Edition European Pharmacopoeia (Supplement), pp. 265–269, Maisonneuve SA, Paris, 2000.*
6) *Kirkwood, TBL, Snape TJ: Biometric principles in clotting and clot lysis assays. Clin lab Haematol, 2: 155–167, 1980.*
7) *Williams KN, Davidson JMF, Ingram GIC: A computer program for the analysis of parallel-line Bioassays of clotting factors. Br J Haematol, 31: 13–23, 1975.*
8) *Counts RB, Hayes JE: A computer program for analysis of clotting factor assays and other parallel-line bioassay. Am J Clin Path, 71: 167–171, 1979.*
9) *Lawrie AS, et al.: Production of a discriminant function for the detection of lupus anticoagulant. Sysmex J Int, 6: 164–167, 1996.*