

## Technical method

### Sensitive and rapid measurement of fibrin polymerisation by laser nephelometry

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Ever since the description by Green<sup>1</sup> of a modification of the colorimetric method reported by Soria<sup>2</sup> to determine the kinetics of fibrin polymerisation induced by reptilase was published, no attempts have been made to improve the sensitivity of the method.

The controversial association of the abnormalities of fibrin polymerisation detected by traditional methods with the presence and severity of bleeding disorders<sup>1-5</sup> or with the electrophoretic abnormalities of the fibrinogen molecule reported previously,<sup>6</sup> or both, prompted us to search for a more reliable method to measure fibrin polymerisation.

We report on the development and characteristics of a method using laser nephelometry to determine the kinetics of fibrin polymerisation induced by reptilase, as well as its application to the study of patients with chronic liver disease.

#### Material and methods

Thirty six young healthy volunteers were studied. Criteria for the selection of normal donors included a history free of haemorrhagic diathesis and the normality of routine haemostatic tests (bleeding time, prothrombin and partial thromboplastin times, and fibrinogen concentration).<sup>7</sup>

Fifty two patients with confirmed alcoholic liver cirrhosis were also studied, and their serum bilirubin and prothrombin values at the time of the study were recorded, as was the presence and severity of bleeding and encephalopathy. All cirrhotic patients were classified according to their degree of portal hypertension as defined by Child.<sup>8</sup> Venous blood (5 ml) was collected into tubes containing 0.5 ml of 3.13% tri-

sodium citrate solution from all subjects. Samples were processed within three hours, unless otherwise stated.

#### LASER NEPHELOMETRY MEASUREMENT OF FIBRIN POLYMERISATION

Fibrinogen concentration was measured by Quick's method<sup>9</sup> and adjusted in all samples to 0.6 g/l by dilution with 0.15M sodium chloride. Two hundred and fifty microlitre aliquots of each sample were placed in a plastic cuvette, and a voltage reading of the turbidity was recorded in a laser nephelometer (Behring, serial no 19471/678). This value was used as the sample blank for further calculations. Keeping the cuvette inside the well of the nephelometer, 10 µl of a 34 mg/ml solution of reptilase (Bothrops atrox-venom thrombin like fraction, Laboratorios Chinoín, Mexico, lot no 102) was added to the diluted plasma sample: at the same time a stopwatch was started. Voltage values were recorded every 15 seconds for a total of seven minutes, as the phenomenon becomes asymptotic at five minutes. The sample blank was subtracted from all subsequent readings, and a time and voltage linear plot was built for each sample. Normal ranges for each period were established by calculating the mean and variance of the values obtained in the 36 healthy volunteers.

In selected experiments the effect of storage on the samples was evaluated by repeatedly measuring fibrin polymerisation in four samples that were aliquoted and stored either at room temperature, at 4°C, or at -20°C for up to 48 hours. Determinations of samples that had been thawed more than once were not made. In another series of experiments the interassay reproducibility was investigated by measuring fibrin polymerisation 10 times in fresh samples of four healthy donors. Finally, additional experiments in which the content of a single phial of reptilase was used for several days after its reconstitution, to induce fibrin polymerisation in daily fresh samples of two healthy volunteers, were performed to evaluate the stability of the reptilase at 4°C.

#### COLORIMETRIC MEASUREMENT OF FIBRIN POLYMERISATION

This was performed exactly as described by Green,<sup>1</sup> so that it could be used as a reference method.

#### Results

Fig. 1 depicts the mean variance values obtained in the normal control group for each one minute period. Results were expressed as a per cent of aggregation:

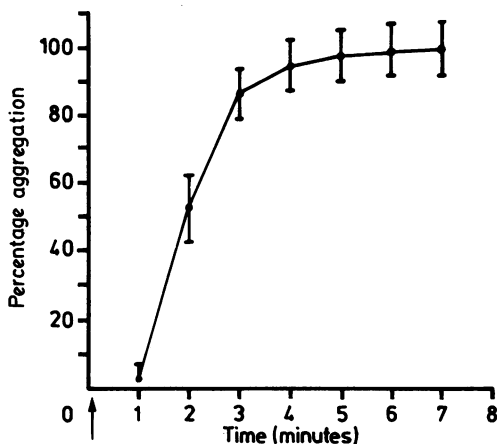


Fig. 1 Results of fibrin polymerisation induced by reptilase determined in 36 young healthy volunteers by laser nephelometric procedure. Arrow indicates time of induction.

100% was the mean value of maximal aggregation of all 36 normal subjects studied. Measurements after seven minutes were performed in the initial experiments, but as the phenomenon became asymptotic at five minutes in all normal instances, these determinations were omitted.

#### COMPARISON OF NEPHELOMETRIC AND COLORIMETRIC ASSAYS

Fig. 2 shows the percentage aggregation values (taking the maximal aggregation as 100%) obtained from 56 determinations at different intervals by both the colorimetric (X-axis) and the nephelometric methods. Results of the linear regression analysis, also shown in Fig. 2, indicate that the nephelometric method detected more initial aggregation earlier and completed aggregation faster than the colorimetric method. In fact, by the end of the seventh minute the colorimetric method detected 86.8 (SD 4.4)% of the maximal aggregation, which can be detected only after 11 minutes by induction.

#### SAMPLE STABILITY

When plasma samples were stored at room temperature no change in the measurements was observed after eight hours, but afterwards a sudden drop in the aggregation occurred, reaching a loss of 50% of the initial values at 24 hours. When samples were stored at 4°C no loss of activity was detected in the first 24 hours, but the activity started to decrease slowly thereafter. At 72 hours the measurable activity was 72% of the initial values.

When samples were stored frozen a similar pattern to that of the refrigerated samples was observed. A slightly higher activity (85%) was observed at 72 hours. No attempts to determine the stability of the samples beyond this period under either circumstance were made.

#### INTERASSAY REPRODUCIBILITY

The evaluation was done as described and showed different degrees of variation throughout the whole seven minute assay. The coefficients of variation were unacceptable (> 10%) for the measurements within the first two minutes of the assay; they were, however, always below 5% afterwards, and less than 2% by the fifth minute, and so on. This high variation in the beginning of the assay seemed to be due to the uneven mixture of the plasma with the (inducer) reptilase. Several ways of mixing both reagents for maximum effect were attempted, but the interassay variation coefficient could not be significantly reduced for the first part of the assay. Hence the results in this part of the assay were disregarded for any evaluation of or comparison with abnormal (patients) samples.

#### REPTILASE STABILITY

Once reconstituted the reptilase solution was capable of inducing the same degree of kinetics and fibrin aggregation for six days. A slow decrease in its ability to induce aggregation occurred subsequently, and a total loss of this property was seen by day 15.

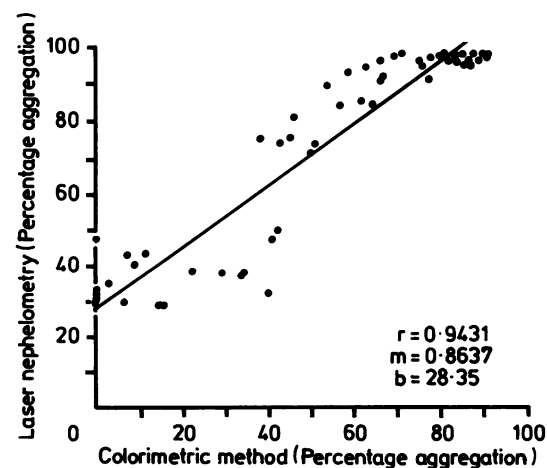


Fig. 2 Scattergram showing fibrin aggregation results obtained by colorimetric and nephelometric methods in 56 simultaneous determinations. Insert gives results of linear regression analysis.

## FINDINGS IN PATIENTS WITH LIVER CIRRHOSIS

Two different types of abnormality were recorded in these patients. One consisted of a diminished (below  $X-2$  (SD) of normal) maximal aggregation of fibrin monomers, while the other was the delayed occurrence of the 50% of the maximal aggregation. The first type of defect was found in 28.8% of the patients (15/52) and the second was evident in 11.6% (6/12); the combined presence of both was seen in 15 of the 52 (28.8%) patients. Therefore, a defect of some kind was documented in 69.2% of the patients.

We found a significantly larger number of patients with severe forms of encephalopathy among those whose maximal fibrin aggregation was decreased than in those with normal results ( $\chi^2 = 7.4677$ ,  $p < 0.05$ ). We also found that a very high proportion of patients with severe encephalopathy had delayed fibrin polymerisation ( $\chi^2 = 14.4667$ ,  $p < 0.0025$ ).

No association was found between the level of maximal aggregation and the presence and severity of bleeding, but severe bleeding was more commonly seen among patients with delayed polymerisation ( $\chi^2 = 4.98$ ,  $p < 0.05$ ).

Association of either defect in polymerisation with the bilirubin concentrations, prothrombin time values, and degree of portal hypertension was evaluated and found to be absent in all instances.

## Discussion

Our findings indicate that the use of laser nephelometry for the measurement of fibrin polymerisation offers several advantages over the traditional colorimetric method as follows: the light source of the nephelometer instrument is a very intense angled collimated laser beam that gives the method a much higher sensitivity when measuring light scattering, compared with spectrophotometers. This permits the much earlier detection of initial (small size polymers) fibrin aggregation, as well as a faster recording of the further formation of the fibrin lattice up to its endpoint.

We observed a higher incidence of abnormal fibrin aggregation among patients with liver cirrhosis than that reported by Green using the traditional colorimetric assay.<sup>1</sup> In some of these patients the abnormality consisted of a delayed, rather than a poor, aggregation of fibrin, which could not be assessed by

the colorimetric method. We believe that these particular patients may have accounted for the difference observed, and conclude, therefore, that the proposed method also bears higher sensitivity for the detection of patients with certain types of previously undescribed fibrin polymerisation abnormalities. Moreover, it is the delayed aggregation defect that was found in association with severe bleeding and has strongly associated with severe forms of encephalopathy.

An additional advantage of the laser nephelometry method is that it uses one tenth of the volume of reptilase as well as that of the plasma sample to be tested, making the procedure particularly suitable for small sample analysis and more economical.

The principal drawback of the assay is the price of the laser nephelometry equipment. The growing number of tests that can be undertaken with its use, however, justifies its purchase.

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