A simple quantitative assay of tissue plasminogen activator

LINDA DE COSSART, RW MARCUSON

From the Department of Surgery, Hope Hospital, Eccles Old Road, Salford M6 8HD

SUMMARY A simple method for the quantitative assay of tissue plasminogen activator is described. Human veins and uterus obtained at operation are disintegrated in a membrane disintegrator at -70°C and a known weight of the powder, suspended in buffered saline and thoroughly mixed. Assay of the dilutions of this homogenate on isotope-labelled fibrin clots gives straight line plots of log weight against log activity of the dilution and the sample activity is calculated from this.

The method has been compared with the conventional histochemical technique. A highly significant correlation between the results of the two methods has been obtained ($r = 0.79$; $p < 0.001$).

The principal methods currently used to measure plasminogen activator in tissue are the potassium thiocyanate extraction technique¹ and the histochemical fibrin slide method first described by Todd² and modified by Pandolfi *et al.*³ The latter method has been used most commonly to study the relation between disease and tissue plasminogen activator⁴⁻¹⁰ but despite various scoring systems remains semiquantitative. We have developed a more quantitative technique in which dilutions from homogenates of tissue are assayed on fibrin clots labelled with ^{125}I fibrinogen. The results have been compared with those obtained by the histochemical method.

Material and methods

Samples of vein and uterus obtained at operation or after amputation were immediately rinsed in buffered saline at 4°C to remove any blood contamination. They were dried on filter paper and carefully divided into two pieces which were frozen to -70°C without delay. One piece was kept at -70°C until assayed by an homogenate technique. The other piece was frozen onto a chuck immersed in liquid nitrogen using mounting medium. This chuck was kept in a sealed container in a vacuum flask containing cardice in a -70°C freezer until assayed by the histochemical technique described by Pandolfi *et al.*³ modified as described below.

HOMOGENATE TECHNIQUE

A weighed sample of the tissue was disintegrated in a Mikrodismembrator II (B Braun) at -70°C for 25 s. The powdered remains were resuspended in 2 ml buffered saline at 4°C and mixed thoroughly. Four further dilutions of this homogenate were made to give five aliquots of dilution 1/1, 1/2, 1/4, 1/8, 1/16 (wt/vol). Two 30 μl aliquots of each dilution were placed on heated and unheated fibrin clots prepared as follows:

Unheated

Human fibrinogen grade L (Kabi) was dissolved in Michaelis-buffered saline (pH 7.4) to a working solution of 0.01% wt/vol. ^{125}I fibrinogen 110 μC was dissolved in 2 ml of the buffered saline in a sealed container. This was added to the fibrinogen solution to give a working concentration of 0.05 $\mu\text{C}/\text{ml}$. Of this solution 10 ml were clotted in a 9 cm Petri dish (Sterilin) with 0.5 ml of a solution of calcium thrombin (Leo) 20 U/ml. The plates were allowed to consolidate at room temperature for one hour. The same batch of fibrin was used for all assays. After inoculation with the dilutions of the homogenate the fibrin plates were incubated on a level surface at 37°C for 22 h. A 30 μl sample of urokinase (Leo) 50 U/ml was placed on each fibrin plate to ensure similarity of the counts released by each fibrin plate. Even distribution of the ^{125}I fibrinogen was checked by assay of 10 samples of urokinase on these plates freshly prepared (5975 ± 244) and after two weeks

storage (5023 ± 115). Adjustment was made for any difference in counts.

Heated

Heated plates were similarly prepared but in a glass Petri dish to allow heating in an oven at 80°C for 30 min to destroy plasminogen according to the method described by Lassen.¹

After incubation the volume of fibrin lysed was aspirated into a counting tube and the hole in the fibrin washed with 1 ml of water which was also placed in the counting tube. The tube was counted in a gamma counter for 10 min. The activity of the aliquot was expressed as the mean of the corrected counts per minute released by each $30\ \mu\text{l}$ sample.

Regression lines for the log cpm and the log weight of vein (μg) were drawn for each dilution. The activity score of the specimens was calculated from the graph and was equivalent to the counts released by one microgram of tissue. The regression lines of a high activity sample and a low activity sample are shown in Fig. 1. The activity score for each specimen is the intercept of the regression line on the "y" axis.

HISTOCHEMICAL TECHNIQUE

The method described by Pandolfi *et al*³ was used with the following modifications: 0.06 ml of the fibrinogen solution and 0.01 ml of the thrombin solution were mixed on a microscope slide and spread over an area of $10\ \text{cm}^2$. The slides were allowed to consolidate in a moist chamber for 30 min and used within one hour of preparation. Four $8\text{--}10\ \mu\text{m}$ frozen sections were placed on each fibrin slide and five

Fig. 1 The regression lines of log weight against log cpm of two samples A and B. The activity score is the intercept on the "y" axis.

Table 1 Homogenate results in specimens studied

Specimen	Score*
Top long saphenous vein	1948
Top long saphenous vein	1943
Superficial inferior epigastric	1763
Body of uterus	1593
Top long saphenous vein	1520
Top long saphenous vein	1400
Calf vein saphenous specimen	1378
Body of uterus	1375
Top long saphenous vein	1012
Calf long saphenous vein (amputation)	999
Calf varicose vein	850
Amputation long saphenous vein	841
Amputation long saphenous vein	705
Top long saphenous vein	664
Amputation long saphenous vein	643
Amputation long saphenous vein	404
Amputation long saphenous vein	363
Amputation long saphenous vein	328
Calf varicose vein	316
Amputation long saphenous vein	315
Calf varicose vein	144
Amputation calf long saphenous vein	40

*Activity score of specimens was calculated from the graph (see Fig. 1) and was equivalent to counts released by $1\ \mu\text{g}$ of tissue.

Fig. 2 Scatter diagram showing the correlation of the homogenate score (y axis) and the histochemical score (x axis). X—X = regression line and $r = 0.79$

fibrin slides were prepared for each specimen. They were incubated in a moist chamber at 37°C for 5, 10, 20 and 30 min. The fifth slide was used as a control and was placed in the formalin without incubation. Scoring was as follows: 0 = no lysis; 1 = pinhole lysis; 2 = distinct areas of lysis; 3 = large areas of lysis but no coalescence; 4 = massive lysis. The mean score for each slide was determined and the

Table 2 Results of the two assays on the long saphenous vein from the leg obtained immediately after amputation

Specimen	Homogenate score*	Histochemical score
1	328	2
2	841	2
3	643	2
4	999	2
5	404	2

*See footnote to Table 1.

activity of the specimen was the cumulative score for each sample.

Results

Twenty samples of vein from different sites and two specimens of uterus were examined by both methods. Table 1 shows the results obtained by the homogenate technique and the site of origin of the veins.

The correlation of the results obtained by the two methods is shown in Fig. 2. The correlation coefficient $r = 0.79$.

A range of activity was apparent from the homogenate score which was not indicated by the histochemical score. This is demonstrated in Table 2 by the results of the assays on adjacent pairs of samples taken from five sites on a single long saphenous vein removed from the calf of an amputated limb.

There was no lysis on any of the heated plates.

Discussion

A new method for measuring plasminogen activator in tissue has been described and the results obtained compared with those by an established technique. A highly significant correlation between the two sets of results has been demonstrated.

The homogenate technique has several advantages in that it is simple to perform, is technically easier than the histochemical method, is quantitative and free from subjective error. Venous tissue is difficult to homogenise in a Potter's homogeniser as used in the potassium thiocyanate technique but the Mikro dismembranator II, a percussive ball homogeniser, is particularly good for this. It is often used for the preparation of tissue samples for analysis by infrared, x-ray, and electron spectroscopy. Disintegration takes place at -70°C which makes the tissue brittle and ensures the tissue is kept at low temperature during the procedures. Samples of vein (20–40 mg) are disintegrated in 25 s and we have confirmed by electron microscopy of the debris that complete breakdown of the cell is achieved. The

homogenate is easily suspended in buffered saline.

The histochemical technique gives useful information on the site of activity of the plasminogen activator and has been widely used in clinical studies in veins.^{4–7} Nevertheless, the method requires considerable expertise to produce satisfactory results and remains semiquantitative. No results of assay of small veins by the potassium thiocyanate extraction technique have been reported.

Johnson and Mansfield¹² attempted to quantify activator release from whole vein by incubation of the vein endothelial surface down on an ^{125}I fibrin clot. Their method however does not correlate with the results by the histochemical technique.¹³

Using the homogenate technique we have been able to quantify plasminogen activator in various tissues and have shown that thigh long saphenous vein has considerably higher activity than calf long saphenous vein (unpublished data).

We conclude therefore that this new homogenate technique is a useful and viable method for the investigation of tissue plasminogen activator.

This work was made possible by a grant from the Manchester Regional Health Authority. We are grateful to Professor Miles Irving for the use of facilities in the Department of Surgery, Hope Hospital. We are especially grateful to Dr Jean Thomson, Withington Hospital, who gave invaluable help in preparing this paper.

References

- 1 Astrup T, Albrechtsen OK. Estimation of the plasminogen activator and the trypsin inhibitor in animal and human tissue. *Scand J Clin Lab Invest* 1957;9:233–43.
- 2 Todd AS. Fibrinolysis autographs. *Nature* 1958;181:495–6.
- 3 Pandolfi M, Nilsson IM, Robertson B, Isaacson S. Fibrinolytic activity of human veins. *Lancet* 1967;2:127–8.
- 4 Astedt B. Low fibrinolytic activity of veins during treatment with oestrin. *Acta Obstet Gynaecol Scand* 1971;50:279–83.
- 5 Browse NL, Gray L, Jarrett PEM, Morland M. Blood and vein wall fibrinolytic activity in health and vascular disease. *Br Med J* 1977;i:478–81.
- 6 Isaacson S, Nilsson IM. Defective fibrinolysis in blood and vein walls in recurrent idiopathic venous thrombosis. *Acta Chir Scand* 1972;138:313–9.
- 7 Nicolaides AN, Clark CT, Thomas RD, Lewis JD. Fibrinolytic activator on the endothelium of the veins in the lower limb. *Br J Surg* 1976;63:881–4.
- 8 Cunliffe WJ, Dodman BA, Dodman EA, Webster J. A method for the quantitative assessment of tissue fibrinolysis. In: Davidson JF, Samama M, Desnoyers PC eds. *Progress in chemical fibrinolysis and thrombolysis*. Vol 1. New York, Raven Press: 1975:241–7.
- 9 Kwaan HC, Astrup T. Demonstration of fibrinolytic activity by the histochemical fibrin slide technique. *Lab Invest* 1967;17:140–4.

- ¹⁰ Kwaan HC, Astrup T. Localisation of fibrinolytic activity in the eye. *Arch Pathol* 1963;**76**:596–601.
- ¹¹ Lassen M. Heat denaturation of plasminogen in the fibrin plate method. *Acta Physiol Scand* 1952;**27**:371–6.
- ¹² Johnson RH, Mansfield AO. A new method for the detection of the plasminogen activator content of vein walls. *Acta Haematol* 1978;**60**:243–9.
- ¹³ Marsh NA, Gaffney PJ. The rapid fibrin plate—a method for plasminogen activator assay. *Thromb Haemost* 1977;**238**:545–51.

Requests for reprints to: Dr Linda de Cossart, Department of Surgery, Hope Hospital, Eccles Old Road, Salford M6 8HD, England.