A simple quantitative assay of tissue plasminogen activator

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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 $-70^\circ$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 technique$^1$ and the histochemical fibrin slide method first described by Todd$^2$ and modified by Pandolfi et al.$^3$ The latter method has been used most commonly to study the relation between disease and tissue plasminogen activator$^4$--$^10$ 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 $4^\circ$C to avoid any blood contamination. They were dried on filter paper and carefully divided into two pieces which were frozen to $-70^\circ$C without delay. One piece was kept at $-70^\circ$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 $-70^\circ$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 $-70^\circ$C for 25 s. The powdered remains were resuspended in 2 ml buffered saline at $4^\circ$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 $\mu$l 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 $\mu$C 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 $\mu$C/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 $\mu$l 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'

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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.

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 μl sample.

Regression lines for the log ccppm 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 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 cm². The slides were allowed to consolidate in a moist chamber for 30 min and used within one hour of preparation. Four 8–10 μm frozen sections were placed on each fibrin slide and five 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>
<thead>
<tr>
<th>Specimen</th>
<th>Score*</th>
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<tbody>
<tr>
<td>Top long saphenous vein</td>
<td>1948</td>
</tr>
<tr>
<td>Top long saphenous vein</td>
<td>1943</td>
</tr>
<tr>
<td>Superficial inferior epigastric</td>
<td>1763</td>
</tr>
<tr>
<td>Body of uterus</td>
<td>1593</td>
</tr>
<tr>
<td>Top long saphenous vein</td>
<td>1400</td>
</tr>
<tr>
<td>Top long saphenous vein</td>
<td>1378</td>
</tr>
<tr>
<td>Calf vein saphenous specimen</td>
<td>1375</td>
</tr>
<tr>
<td>Body of uterus</td>
<td>1012</td>
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<tr>
<td>Top long saphenous vein</td>
<td>999</td>
</tr>
<tr>
<td>Calf long saphenous vein (amputation)</td>
<td>850</td>
</tr>
<tr>
<td>Calf varicose vein</td>
<td>841</td>
</tr>
<tr>
<td>Amputation long saphenous vein</td>
<td>705</td>
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<tr>
<td>Amputation long saphenous vein</td>
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<tr>
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<td>643</td>
</tr>
<tr>
<td>Amputation long saphenous vein</td>
<td>404</td>
</tr>
<tr>
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</tr>
<tr>
<td>Amputation long saphenous vein</td>
<td>328</td>
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<tr>
<td>Calf varicose vein</td>
<td>316</td>
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<tr>
<td>Amputation long saphenous vein</td>
<td>315</td>
</tr>
<tr>
<td>Calf varicose vein</td>
<td>144</td>
</tr>
<tr>
<td>Amputation calf long saphenous vein</td>
<td>40</td>
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</table>

*Activity score of specimens was calculated from the graph (see Fig. 1) and was equivalent to counts released by 1 μg of tissue.

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

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
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 $-70^\circ$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. 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 Mansfield 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.

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.

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References


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

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Homogenate score*</th>
<th>Histochemical score</th>
</tr>
</thead>
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<tr>
<td>1</td>
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<td>2</td>
</tr>
<tr>
<td>2</td>
<td>841</td>
<td>2</td>
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<tr>
<td>3</td>
<td>643</td>
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<tr>
<td>4</td>
<td>999</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>404</td>
<td>2</td>
</tr>
</tbody>
</table>

*See footnote to Table 1.
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