Technical method

In vitro assay of thrombolysis with ^{125}I -fibrinogen

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The use of radioiodinated fibrinogen for the in vitro testing of plasmin activity was initially reported by Shulman and Tagnon.¹ Investigators have subsequently used radioiodinated fibrinogen to investigate the fibrinolytic enzyme system and to assay fibrinolytic activity in vitro,²⁻⁴ and in vivo.^{5,6} The principle for the measurement of fibrinolytic activity using radiolabelled fibrinogen is based on the rate of release of radioactivity from the digested ^{125}I or ^{131}I -fibrin.

This report describes a radioactive method for assaying thrombolysis in vitro.⁷ It was found to be more sensitive, accurate and convenient than the present assay method,⁸ which is based on the weighing of fixed thrombi after treatment with thrombolytic agents.

Material and methods

RADIOLABELLING OF THROMBI

Radionuclide-labelled ^{125}I -fibrinogen (Ibrin, Amersham), which contained $160\ \mu\text{Ci}$ and $1\ \text{mg}$ of human fibrinogen in a vial, was used to label the thrombi. Artificial thrombi were made from citrated blood containing a trace of ^{125}I -fibrinogen, according to Chandler's loop method⁷ using Connor and Poole's modified apparatus.⁹ In the thrombolytic experiments described below, $70\ \text{ml}$ of venous blood were drawn from a healthy donor via a silicone-coated needle into a polypropylene syringe containing $7\ \text{ml}$ of 3.8% sodium citrate and $10\ \mu\text{Ci}$ of ^{125}I -fibrinogen. Blood, sodium citrate and ^{125}I -fibrinogen were immediately mixed. Experiments were conducted within $10\ \text{min}$ of collection. One ml of the blood was transferred to a $27\ \text{cm}$ length of polyethylene tubing (PE-350, $0.3\ \text{cm}$ ID, Clay Adams) into which was added $0.1\ \text{ml}$ of $0.25\ \text{M}$ CaCl_2 . A loop was formed by connecting both ends of the tubing with a short collar of tubing, and it was rotated at $12\ \text{rpm}$ for $30\ \text{min}$ at 37°C to form a ^{125}I -labelled thrombus. The process was repeated for the number of loops required. The apparatus shown in Fig. 1 can accommodate 80 loops.

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RADIOACTIVE METHOD FOR MEASURING THROMBOLYSIS

After sampling $10\ \mu\text{l}$ of residual blood (blood₁) from each loop, $0.1\ \text{ml}$ of a solution of the thrombolytic agent is added and the loop containing the radio-labelled thrombus is rotated for a given period. If desired, samples of blood can be withdrawn periodically to determine progression of lysis as illustrated in the second experiment described below. Upon completion of the rotation period, the contents of each loop are emptied into a test tube. The residual blood is then decanted and the thrombus is transferred to an aluminium dish and rinsed with 0.9% saline to remove residual blood from the thrombus surface. At the same time, $10\ \mu\text{l}$ of the decanted residual blood (blood₂) is sampled. The radioactivities of the blood samples and the thrombus are then measured by means of a gamma scintillation counter. Thrombolysis is calculated according to the formula given below:

$$\% \text{ lysis} = \frac{\text{Total cpm released into blood from original } ^{125}\text{I-thrombus}}{\text{cpm of original } ^{125}\text{I-thrombus}} \times 100$$

Radioactivity (cpm) of original ^{125}I -thrombus = cpm of partially lysed thrombus plus total cpm released into blood from original ^{125}I -thrombus [A]
[A] is calculated from:

$$\frac{\text{cpm of blood}_2 \times \text{total volume } (\mu\text{l}) \text{ in loop}}{10\ \mu\text{l}} \\ \text{minus } \frac{\text{cpm of blood}_1 \times \text{total volume } (\mu\text{l}) \text{ in loop}}{10\ \mu\text{l}}$$

COMPARISON OF WEIGHING AND RADIOACTIVE METHODS

In this experiment, ^{125}I -thrombi were divided into four groups: saline, and urokinase (UK) in final concentrations of $37.5\ \text{IU}$, $75.0\ \text{IU}$ and $150.0\ \text{IU/ml}$ of blood, respectively (high molecular weight lyophilised urinary UK was kindly supplied by Green Cross Corp). Nineteen thrombi were in the saline control group and 9 in each of the UK groups. After sampling $10\ \mu\text{l}$ of blood (blood₁) from each loop, $0.1\ \text{ml}$ of saline or urokinase solution in normal saline was added. The loops were rotated at $12\ \text{rpm}$, for $4\ \text{h}$ at 37°C . Samples ($10\ \mu\text{l}$) were drawn from the residual blood (blood₂) from each loop and the separated thrombi were rinsed in saline. Fixation of the thrombi in Bouin's solution followed. After $16\ \text{h}$, the thrombi were removed from the fixative,

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Fig. 1 Apparatus for *in vitro* thrombosis housed in acrylic plastic-enclosed 37°C incubator. Up to 80 loops can be mounted on spindle and rotated to form thrombi.

blotted and weighed. The radioactivities of the fixed thrombi and blood samples (blood₁ and blood₂) were measured and the percent lysis was calculated according to the formula given above. Calculation of the percent lysis of a thrombus by the weighing method was performed according to the equation given below:

% lysis = $\left(1 - \frac{\text{Weight of treated thrombus}}{\text{Mean weight of control thrombi}}\right) \times 100$

TIME-COURSE OF THROMBOLYSIS

In this experiment, two ¹²⁵I-thrombi were rinsed with saline and their radioactivities were counted. They were then separately transferred to new loops into each of which was added one ml of fresh citrated blood followed by 0.1 ml of normal saline in one loop and 0.1 ml of urokinase solution in the other loop. The loops were rotated at 12 rpm for 4 h at 37°C. During rotation a 10 µl blood sample was taken at 1, 2, 3, and 4 h after the addition of UK or saline. At the end of 4 h, the radioactivities of the blood samples and each thrombus were measured.

Results

COMPARISON OF WEIGHING AND RADIOACTIVE METHODS

Table 1 shows the mean radioactivity (cpm) and

weight (mg) of the thrombi in the saline group. The coefficient of variability for cpm was 5.2%, or half of the value for mg weight. The correlation between cpm and mg was - 0.13, suggesting no correlation between cpm and mg weight of each thrombus.

Table 2 shows the mean radioactivity (cpm), weight (mg) and % lysis by the radioactive (RA) and weighing (WT) methods for partially lysed thrombi treated with urokinase for 4 h at final concentrations of 37.5 IU/ml, 75.0 IU/ml and 150.0 IU/ml, respectively. The mean weight of the saline thrombi, as shown in Table 1, was used as a control value for calculating percent lysis of the UK groups according to the weighing method.

Table 1 Comparison of mean radioactivity (cpm) and weight (mg) of thrombi in a saline control group

	Mean radioactivity (cpm)	Mean weight (mg)
No thrombi	19	19
Mean	81060	43.87
Range	75613-92884	36.29-49.87
SD	4172	4.46
c	5.2%	10.2%
r	- 0.13	

SD = standard deviation; c = coefficient of variability; r = correlation coefficient.

Table 2 Comparison of radioactive (RA) and weighing (WT) methods for estimating thrombolysis in vitro induced by urokinase (UK)

37.5 IU/ml UK				75.0 IU/ml UK				150.0 IU/ml UK				
Thrombi (n = 9)		% lysis		Thrombi (n = 9)		% lysis		Thrombi (n = 9)		% lysis		
cpm	mg	RA	WT*	cpm	mg	RA	WT*	cpm	mg	RA	WT*	
Mean	72303	43.0	16.5	1.9	30403	14.7	67.0	66.5	12912	5.9	85.9	
Range	61072-83293	38.4-47.2	11.7-23.5	-7.6-12.4	26977-36110	11.7-17.5	63.1-71.2	60.1-73.4	10208-16994	3.0-9.8	81.2-89.5	
SD	7035	3.6	4.4	8.2	3398	2.3	2.7	5.1	2401	2.4	3.2	
c	9.7%	8.4%	26.6%	436.4%	11.2%	15.3%	4.0%	7.7%	18.6%	40.7%	3.7%	
r	0.44†		0.03‡		0.67‡		-0.04†		0.94§		0.84§	

SD = standard deviation; c = coefficient of variability; r = correlation coefficient.

*Mean control weight is mean of saline group in Table 1 = 43.87 mg.

†p > 0.05 ‡p ≤ 0.05 §p < 0.001.

Since the weights of some thrombi in the low UK concentration group of 37.5 IU/ml were larger than the control mean, their percent lysis, as calculated by the weighing method, resulted in negative values. These negative values resulted in a correlation coefficient of 0.03 and a wide spread in the mean percent lysis between the two methods; 1.9% lysis by WT (SD 8.2) and 16.5% lysis by RA (SD 4.4). In the other two UK groups, 75.0 IU/ml and 150.0 IU/ml, the mean values of percent lysis between the two methods were nearly equal (Fig. 2). However, the correlation coefficient for the UK group of 75.0 IU/ml was -0.04; only in the UK group of 150 IU/ml was there a significant correlation coefficient, which was 0.84. The standard deviations of the radioactive method for percent lysis were almost half those of the weighing method at each UK concentration.

TIME-COURSE OF THROMBOLYSIS

Table 3 shows the time-course of UK-induced thrombolysis determined by means of the radioactive method. In the saline control, the sum total radioactivity of the blood at each sampling interval of 1 h and the partially lysed thrombus after 4 h of incubation was nearly equal to the radioactivity of the original thrombus. Similarly, the sum total radioactivity of the blood and the lysed thrombus after 4 h incubation was almost the same as the

Fig. 2 Mean values of percent lysis of thrombi induced by urokinase as calculated by the radioactive and weighing method.

radioactivity of the original thrombus before treatment with urokinase.

Discussion

The radioactive procedure here described provides a sensitive assay for thrombolysis in vitro. The previously used weighing method is not sensitive

Table 3 Time-course of thrombolysis by urokinase using the radioactive method

Group	Total radioactivity (cpm)						Thrombus after 4 h
	Original thrombus	Residual blood					
		0 h	1 h	2 h	3 h		
Urokinase	193 479	720	138 160	156 000	160 520	181 320	5676
150 IU/ml	100%	0.37%	71.4%	80.6%	83.0%	93.7%	2.9%
Saline	220 400	1700	1500	1900	2400	3000	220 753
control	100%	0.77%	0.68%	0.86%	1.09%	1.36%	100.2%

Percentages indicate percent of total radioactivity of original thrombus.

enough to detect slight lysis resulting from low concentrations of lytic agents since individual variation in the control group may at times give lower weights than the experimental group. On the other hand, there is good correlation between the two procedures under conditions of greater lysis. The weighing method reflects, not only lysed fibrin, but also the release of materials from the thrombus other than fibrin. Since fibrinolysis is the process of chief interest in the study of thrombolysis, the radioactive assay, by virtue of its inherent mechanism, would seem to be the most appropriate. With this assay method, the test thrombus serves as its own control so that the time-course and kinetics of thrombolysis can be readily measured over a predetermined schedule. It is concluded that the radioactive method is a reliable, sensitive and rapid procedure for estimating thrombolysis in vitro.

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