Conditions for collection of serum samples for the measurement of fibrin(ogen) degradation products by radioimmunoassay of fragment E

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SYNOPSIS A number of conditions have been assessed for the collection of serum samples for the measurement of fibrin(ogen) degradation products using a radioimmunoassay for degradation fragment E, which permits precise quantitation of differences. Blood should be collected using minimal venous occlusion into glass tubes containing 10 mg/ml of epsilon amino caproic acid and allowed to clot at 4 to 20°C for at least 4 hours before centrifugation. The serum may be stored at 4 or -20°C. Samples from patients receiving anticoagulant therapy should be treated with 10 IU of thrombin per ml.

Serum levels of fibrin(ogen) degradation products (FDP) have been used since 1961 (Ferreira and Murat, 1961) in the diagnosis and treatment of both disseminated and local intravascular coagulation. Fibrinogen and fibrin are degraded by the fibrinolytic enzyme system to produce a heterogeneous series of degradation products of decreasing molecular weight: only the earliest products in the chain retain the ability to clot (Marder *et al*, 1969). When FDP levels are measured in serum only the non-clottable molecules will be present, all others having been removed during coagulation. In preparing serum samples for the measurement of FDP it is essential to ensure that coagulation is complete; if it is not, the measured FDP levels will be falsely raised due to the persistence of clottable molecules. It is equally important to prevent activation of the fibrinolytic enzyme system during coagulation as this could also lead to an increase in levels *in vitro*. With these problems in mind, several workers have suggested conditions for the collection and preparation of serum samples for FDP determinations (table I).

FDP levels can be measured by a variety of immunological techniques including haemagglutination inhibition (Merskey *et al*, 1966), latex particle agglutination (Allington, 1971), immunoelectrophoresis (Niléhn and Nillson, 1964), and

radioimmunoassay (Gordon *et al*, 1973; 1975a; Plow and Edgington, 1973). The radioimmunoassay method has the advantages of high sensitivity and precision, and is thus well suited to the study of variables in sample collection and preparation. In the present study a number of these variables have been examined in detail.

Material and Methods

All blood samples were collected into plastic syringes using minimal venous occlusion. The samples were aliquoted into glass tubes containing either (1) no additive, (2) 10 mg/ml amino caproic acid solution (EACA, Kabi Pharmaceuticals), (3) 2500 Kallikrein inactivator units/ml aprotinin solution (Trasylol, Bayer Pharmaceuticals), (4) 10 mg/ml freeze-dried soybean trypsin inhibitor and 10 NIH units/ml thrombin (Wellcome Reagents), (5) 10 NIH units/ml of freshly suspended thrombin (Parke Davis Ltd) and 10 mg/ml EACA, (6) as (5) but stored at 20°C for 7 days, (7) as (5) but freeze-dried before use. Thrombin was also added to the serum after separation. Incubation times of whole blood from 4 to 96 hours were studied at 4°C, and temperatures from 4°C to 37°C were studied for a fixed time of 24 hours. Serum was separated by centrifugation at 1500 *g* for 45 minutes and stored at either 4°C or -20°C for up to 10 days. In one set of experiments the coagulation

Reference	Method of Venepuncture	Added Thrombin (NIH units/ml)	Added Enzyme Inhibitor	Time for Coagulation (h)	Temperature for Coagulation (°C)	Storage of Separated Serum (°C)
Bonnar <i>et al</i> (1969)	Clean	—	Tranexamic acid 0.2 mg/ml	ns	ns	ns
Henderson <i>et al</i> (1970)	ns	10	—	2	37	—20
Stiehm <i>et al</i> (1970)	ns	—	—	1–3	ns	ns
Almér <i>et al</i> (1972)	ns	10	EACA 8 mg/ml	ns	ns	ns
Briggs <i>et al</i> (1972)	ns	—	Tranexamic acid 0.4 mg/ml	4	20	ns
Merskey <i>et al</i> (1972)	Minimum venous occlusion	1–5	EACA 0.05M	0.5–4	0–21	0 or —20
Bonnar (1973)	ns	5	Aprotinin 250 KIU/ml	2–8	37	—20
Hyde <i>et al</i> (1973)	Careful atraumatic	2	EACA 4 mg/ml	2	37	ns
Naish <i>et al</i> (1973)	ns	5	EACA 0.2M	3	37	—20
Satterwhite <i>et al</i> (1973)	ns	10	EACA 4 mg/ml	2	37	ns
Wellcome Reagents Ltd. (1973)	No venous occlusion	10	Soybeant trypsin inhibitor 0.8 mg/ml	0.5	20–37	ns
Gaffney <i>et al</i> (1974)	ns	2.5	Aprotinin 100 KIU/ml	1	37	ns

Table I Methods of collection of serum samples for FDP assays

ns = not stated

process was interrupted in the presence of EACA (10 mg/ml) by the addition of heparin 25 IU/ml (Weddel Pharmaceuticals) and immediate separation of the blood.

The FgE radioimmunoassay was performed as described previously (Gordon *et al*, 1975a). All the samples were assayed in duplicate at a 1:10 dilution, and EACA, aprotinin, soybean trypsin inhibitor, heparin, and thrombin were all tested for their ability to inhibit antigen-antibody binding. Three quality control sera were included in each assay performed, and comparative samples were always assayed in the same run. Results were compared by linear least squares regression analysis.

Results

When the coagulation process was interrupted by the addition of heparin there was a progressive decrease in FgE immunoreactive material in blood beginning within 10 minutes of exposure to glass by 20°C; by 30 minutes only 0.4% of the initial activity was present at both 4°C and room temperature, and a further decline to 0.1% occurred by 2 hours (fig 1). Incubation of whole blood at 37°C in the absence of EACA resulted in an increase in FgE reactive material within 4 hours (fig 2). The results of the other studies are summarized in table II; incubation for longer than 4 hours at 4°C had no effect on FgE levels; at 37°C there was a significant rise at 24 hours. This rise was greater in the presence of aprotinin, and an even greater increase was seen with soybean trypsin inhibitor in spite of

the presence of thrombin (fig 3). The addition of freshly suspended thrombin, either before (fig 4) or after centrifugation, caused an equal and significant reduction in FgE levels. The addition of

Fig 1 Progressive decrease in FgE related antigen with time during coagulation at 4°C (—) and room temperature (---).

Fig 2 Effect of time on serum FgE levels of whole blood stored at 37°C in the absence of fibrinolytic enzyme inhibitors.

stored thrombin solution had no effect on results whereas freeze-dried material caused a significant increase in FgE levels. There was no difference in results between serum stored at 4°C or -20°C to 10 days. No inhibition of antigen-antibody binding

was observed with EACA, aprotinin, soybean trypsin inhibitor, heparin or thrombin at the concentrations used for the collection of serum.

Fig 3 Serum FgE levels (ng/ml) in samples collected in the presence of EACA or soybean trypsin inhibitor: $r = 0.84$, slope 0.96 ($P > 0.1$), intercept 56 ($0.02 > P > 0.01$).

Parameter tested	Experiment	No. of Subjects	Correlation Coefficient	Slope	Intercept	Difference (%) from Control FgE Value of 200 ng/ml
Incubation time at 4°C before separation (EACA present)	4 h (control 24 h)	10	0.93	0.99	11	0
	96 h (control 24 h)	13	0.90	0.88	-1	0
Incubation temperature for 24 h before separation (EACA present)	20°C (control 4°C)	20	0.91	1.20	36	0
	37°C (control 4°C)	58	0.98	1.01	14***	+7.5
Enzyme inhibition	Aprotinin (control EACA)	30	0.93	1.09	32**	+13
	Soybean trypsin inhibitor (control EACA)	37	0.84	0.96	56***	+25
Thrombin before separation (EACA present)	Freshly suspended (control no thrombin)	89	0.99	0.90*	-5	-15
	Suspended and stored at 20°C before use (control no thrombin)	38	0.88	0.90	1	0
	Freeze-dried before use (control no thrombin)	34	0.95	1.23*	-33****	+8.5
Thrombin (after separation EACA present)	Freshly suspended (control no thrombin)	44	0.88	0.63*	46****	-16
Storage temperature after separation (EACA present)	4°C (control -20°C)	26	0.91	0.99	15	0

Table II A comparison of serum FgE levels using a variety of conditions for the collection of serum samples

Significant difference of the slope from 1: *0.005 > P > 0.001

Significant difference of intercept from 0: ** 0.05 > P > 0.025

*** 0.02 > P > 0.01

**** 0.01 > P > 0.005

Fig 4 Serum FgE levels (ng/ml) in samples collected in the presence of EACA with or without freshly suspended thrombin: $r = 0.99$, slope 0.90 ($0.005 > P > 0.001$), intercept -5 ($P > 0.1$).

Discussion

There is only one previous study in which the optimal conditions for the collection of serum samples for FDP assay have been examined (Merskey *et al*, 1972). In the present investigation a variety of conditions have been evaluated in detail. To be of diagnostic value FDP levels in serum should reflect circulating degradation products and not an artefact due to either incomplete fibrinogen conversion or to activation of the fibrinolytic system. Activation of the coagulation system *in vitro* by contact with glass will activate the fibrinolytic enzyme plasmin. The extent of plasmin action will depend on the degree of activation and the time and temperature exposure. Although naturally occurring plasmin inhibitors are found in blood, plasmin is selectively concentrated within the fibrin mesh and may escape the effects of these inhibitors (McNicol and Douglas, 1972). In addition to plasmin, a number of other proteases including trypsin are able to degrade the fibrin molecule (Latallo *et al*, 1971). However, the results described here would indicate that *in vitro* degradation of fibrin is mainly due to plasmin. Thus EACA, which is a specific plasmin inhibitor (Brockway and Castellino, 1971), almost completely inhibited FDP formation *in vitro*, whereas less specific protease inhibitors (aprotinin and soybean trypsin inhibitor) produced only partial inhibition.

Activation of the fibrinolytic system occurs despite the presence of EACA when whole blood is incubated at 37°C for 24 hours. The presence of fibrin is essential since the effect is not seen with separated serum, as previously shown by Merskey and his coworkers (1972).

Optimal activation of the contact factors (XI, XII) of the coagulation system *in vitro* depends on a number of factors including time, temperature, calcium concentrations, and glass in contact with the blood (Nossel, 1972). In the absence of thrombin, coagulation is 96% complete within 10 minutes at 20°C and 99.9% complete within 60–90 minutes at both 4°C and -20°C . The addition of freshly prepared thrombin produces significant reduction in the final concentration of FDP measured, and it seems likely that the concentration of endogenous thrombin produced *in vitro* is insufficient to coagulate all the clottable fibrinogen molecules. Observations on the nature of FDP in normal subjects (Divall, 1974) suggest that the material measured with immunological techniques consists mainly of soluble fibrin polymer of molecular weight greater than that of fibrinogen. These polymers contain partly degraded fibrinogen as judged by the ability of normal serum to cross react in the radioimmunoassay for the neoantigen of fragment D which is specific for the degradation products of fibrin(ogen) (Gordon *et al*, 1975b). The presence of additional exogenous thrombin probably completes the coagulation of these partially degraded, high molecular weight fragments, whether added before or after separation of the serum. However, from the point of view of routine use, the disadvantage of thrombin addition is that the solution cannot be freeze-dried nor can it be stored at room temperature. The use of thrombin should probably be confined to those patients on anticoagulant therapy. In the present study, the findings of Merskey and his coworkers (1972) that thrombin will cause an elevation of FDP levels has not been confirmed. This discrepancy may perhaps be explained by the lack of sensitivity and precision of the tanned red cell haemagglutination inhibition assay used in the previous study.

Venous occlusion has been shown previously to cause a rise in the serum levels of FgE related to the rise in haematocrit and therefore due to haemoconcentration (Butler *et al*, 1975).

In conclusion, the optimum procedure for the collection of blood for FDP assay is as follows: samples should be taken using minimal venous occlusion into glass tubes containing 10 mg/ml of EACA and allowed to clot at 4°C to 20°C for at least 4 hours before centrifugation. The serum may be stored at either 4°C or -20°C . Samples

from patients receiving anticoagulant therapy should be treated with 10 units of thrombin per ml of blood.

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