Comparison of an immunochemical assay for plasma fibrinogen and a turbidimetric thrombin clotting technique to discriminate hyperlipidaemic patients from healthy controls

M L Knapp, M D Feher, H Carey, P D Mayne

Abstract
Plasma samples from patients attending a lipid clinic (n = 14) and healthy control subjects (n = 21) were assayed for fibrinogen using an immunochemical method (radial immunodiffusion) and a turbidimetric assay based on the thrombin clotting technique. The patients had significantly higher plasma fibrinogen concentrations than controls by both methods, but there was significant overlap between the two groups when fibrinogen was assayed by the thrombin clotting technique; there was almost complete separation of the two groups using the immunochemical assay. This difference in overlap could not be attributed to the presence or absence of fibrinogen degradation products. These findings may have important implications for the choice of method for determining plasma fibrinogen when assays are used for the assessment of cardiovascular risk. It is recommended that plasma fibrinogen should be assayed by both an immunochemical and a thrombin clotting method.

Increased plasma fibrinogen concentration has recently been shown to be an independent risk factor for the development of cardiovascular disease. In diabetic subjects studies have shown an association between increased fibrinogen and microvascular and macrovascular complications. Numerous methods can be used for the estimation of plasma fibrinogen including thrombin clotting techniques, heat precipitation, salt precipitation and immunochemical assay.

With the aim of establishing a plasma fibrinogen method to be used for risk factor analysis in the routine evaluation of patients we compared the result of fibrinogen assayed by a thrombin clotting technique and by an immunochemical method (radial immunodiffusion) in a group of normal healthy controls and hyperlipidaemic patients.

Methods
All reagents were analytical grade unless otherwise stated. Thrombin 5000 NIH units/phiial was obtained from Rorer Pharmaceuticals, Eastbourne, Sussex. Protime sulphate was obtained from BDH Ltd, Poole, Dorset. Phosphate buffered saline (pH 6·4) was prepared by dissolving KH₂PO₄ (0·91 g), Na₂HPO₄ (0·47 g), and NaCl (8·5 g) in distilled water to a final volume of one litre.

Blood was collected from 14 patients attending the lipid clinic at the Westminster Hospital (mean age 63 years, range 49–73 years). Five of the patients were receiving treatment for hypertension and nine had clinical evidence of macrovascular disease. All patients were being treated for hyperlipidaemia (by diet and drugs). No patient had diabetes; one patient was a current smoker. Blood was also collected from 21 healthy normolipidaemic laboratory volunteers aged 20–40 years. None of the volunteers was taking any medication and none had a history of vascular disease. Samples were collected into sodium citrate, 38 g/l (9 volumes of blood per volume of anticoagulant), and centrifuged at 1000 × g for 15 minutes. The plasma was separated and stored within one hour of collection at −40°C until assay. Samples were analysed within one month of collection.

FIBRINOGEN ASSAY
Samples for determination of plasma fibrinogen were assayed by an immunochemical method (radial immunodiffusion) and a turbidimetric method based on the thrombin clotting technique. Samples were thawed at 37°C for one hour before assay. No trace of fibrin clot was detectable by visual inspection in any of the samples.

For radial immunodiffusion, commercially prepared "Partigen" plates were used (Hoechst, Hounslow, Middlesex) Behringwerke plasma protein standard was used as calibrant (Behringwerke AG, Marburg, West Germany). The plates were used according to the manufacturer's instructions. The between plate coefficient of variation was 5-0% at a fibrinogen concentration of 4·1 g/l.

Assay of plasma fibrinogen using the turbidimetric method based on the thrombin clotting technique was carried out on the Centrifichem 600 Centrifugal Analyser (Baker Instruments, Surrey). Pooled normal human plasma collected into sodium citrate was used as standard. The fibrinogen concentration of this standard (3·6 g/l) was assigned by assaying it five times using radial immunodiffusion with the Behringwerke plasma protein standard as calibrant. The clotting reagent contained thrombin (2250 NIH units/l), CaCl₂ (2·5 mmol/l), and protime sulphate (10 mg/l) in phosphate buffered saline, pH 6·4. The thrombin was stored at −40°C as a stock solution of 45 000 NIH units/l in 25% (v/v) glycerol and was added to the clotting reagent just before
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use. Briefly, sample (40 μl) and diluent (40 μl
H2O) was mixed with clotting reagent (180 μl)
at an analyser temperature setting of 37°C. The
increase in absorbance at 340 nm was mon-
tored for between three and 90 seconds and was
linearly related to fibrinogen concentration up
to at least 7 g/l. The between assay coefficient of variation was 4.2% at a fibrinogen concen-
tration of 3.8 g/l.

ASSAY OF FIBRINOGEN DEGRADATION PRODUCTS
The presence or absence of significant amounts of fibrinogen degradation products in the
samples was determined on slides by semiquan-
titative latex agglutination immunoassay.
("Fibrinostrum", Organon Teknika Corpora-
tion, Durham, USA). This assay is specific for
the cross-linked D-dimer domain in fibrin.
If cross-linked D-dimer domain fibrin deriv-
atives are detected in plasma, it confirms that
thrombin was formed and that factor XIII
was activated with reactive in vivo fibrinolysis. 18 19
The detection limit of this assay is 500 μg/l in
fibrinogen concentration equivalents.

Analysis of correlation between the two
methods for plasma fibrinogen assay was car-
ried out by least squares linear regression.
Deming’s correction for slope was not applied
because Sex/Sx was < 0.2.20 The χ2 test for
2 × 2 contingency tables incorporating a
rection for continuity and the Mann–Whit-
ney U test were used to analyse the significance
of any differences between the plasma fibrino-
gen concentrations observed in the two
groups of subjects. The F test was used to
analyse the significance of any differences in
the variance of plasma fibrinogen values be-
tween the groups. 21

Results
There was a good correlation between the
turbidimetric thrombin clotting technique and
radial immunodiffusion for plasma fibrinogen
assay using the samples from the lipid clinic
patients and healthy control subjects (fig 1).

Lipid clinic patients as a group had sig-
cificantly higher plasma fibrinogen concentra-
tions than the healthy control subjects when
plasma fibrinogen was assayed either by the
turbidimetric thrombin clotting technique (p
< 0.01) or radial immunodiffusion (p < 0.001)
(fig 2).

Optimal discrimination of control subjects
from hyperlipidaemic patients was achieved at
a cut off of plasma fibrinogen of 4.5 g/l for the
turbidimetric thrombin clotting technique and
3.5 g/l for radial immunodiffusion. Using
these cut off levels, the discrimination of con-
tral subjects from patients was significantly
better when plasma fibrinogen was assayed by
radial immunodiffusion than when measured by
the turbidimetric thrombin clotting tech-
nique (χ2 test: 0.02 < p < 0.05). Furthermore,
the variance of plasma fibrinogen concentra-
tion observed in the control subjects was sig-
ificantly less using radial immunodiffusion
than by the turbidimetric thrombin clotting
technique (F test: p < 0.05), although no sig-
nificant difference in variance was observed for
the lipid clinic patients (F test: p > 0.05).

Only three out of 35 samples were positive
for fibrinogen degradation products by latex
agglutination immunoassay; one positive
sample was from an apparently healthy control
subject and the remaining two positive samples
were from the patients. All three samples had
fibrinogen degradation products at the limit of
assay detection (500 μg/l).

Discussion
This study has shown that although there is a
significant correlation between the turbidi-
metric clotting technique and radial immu-
nodiffusion for plasma fibrinogen assay, discri-
mination between hyperlipidaemic patients and a group of healthy control subjects is
better by the immunochemical assay. This
may have significant implications for the choice
of method for plasma fibrinogen when assays
are used for the assessment of cardiovascular
risk.

It is unlikely that the improved discrimina-
tion by radial immunodiffusion can be attribu-
justed to differences in the amount of in vivo fibrinolysis as only three samples had detectable fibrin D-dimer domain by latex immunone- assay. It could be argued that the samples from the patients contained more early fibrinogen degradation products (X, Y, and E) which are not detected by the latex immunoneo-assay. If present these products would almost certainly cause overestimation of fibrinogen by radial immunodiffusion because of antigenic cross reaction with the intact fibrinogen molecules. Our data do not support this view, however, because the plasma fibrinogen results by radial immunodiffusion are lower in both healthy controls and hyperlipidaemic patients compared with results using the turbidimetric thrombin clotting technique.

Discrepancies between plasma fibrinogen assay by radial immunodiffusion and the thrombin clotting technique have been previously described following surgery or extensive intravascular thrombosis and during the menstrual cycle. None of the patients in our study, however, had recently had surgery or extensive intravascular thrombosis. During the menstrual phase, immunoreactive fibrinogen is higher than fibrinogen assayed by the thrombin clotting technique because of the increased concentration of circulating fibrinogen fragments generated by the breakdown of the endometrium. In our study concentrations of immunoreactive fibrinogen were actually lower than thrombin clottable fibrinogen in both the patient and control groups which makes it unlikely that there were increased amounts of circulating fibrin fragments in our group of patients or controls.

The difference in the median plasma fibrinogen concentrations between the normal healthy controls and hyperlipidaemic patients was similar and was about 1 g/l. It is the lower variance of fibrinogen values seen in the controls when fibrinogen is assayed by radial immunodiffusion that is the major reason for the improved discrimination between the normal controls and hyperlipidaemic patients when using the radial immunodiffusion assay. It is unlikely that a difference in assay precision could be responsible for this as both methods have similar precision.

Our study needs to be extended to ascertain whether similar discrepancies are observed between immunochromatographic methods and the clot weight assay and with heat or salt precipitation. Our results may indicate that the significance of plasma fibrinogen concentra-
tion as a risk factor for vascular disease may depend on method. Until further work has been carried out, we recommend that for purposes of comparison, plasma fibrinogen should be assayed by both an immunochromatographic and turbidimetric thrombin clotting method.

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6 HANDA K, Kono S, Saku K, et al. Plasma fibrinogen levels as an independent indicator of severity of coronary athero-
10 Schneider CL. Rapid estimation of plasma fibrinogen concen-
tration and its use as a guide to treatment of intravas-
15 RAMPING MW, Gaffney PJ. The sulphate precipitation method for fibrinogen measurement; its use on small samples in the presence of fibrinogen degradation products. Clin Chim Acta 1976;78:43-52.
16 FARREL GW, WOLF P. Immunonephelometric determina-