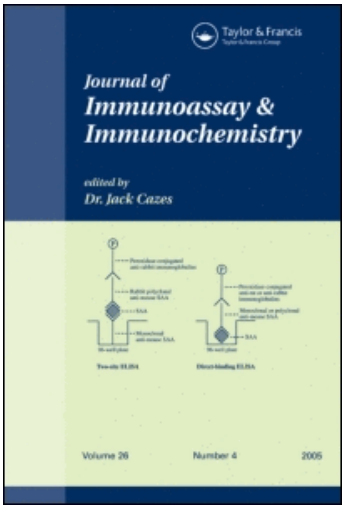

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EVALUATION OF A COMMERCIAL KIT FOR THE RADIOIMMUNOASSAY OF FIBRINOPEPTIDE A

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ABSTRACT

Measurement of fibrinopeptide A (FpA) provides a sensitive and specific marker of thrombin generation and is important in the investigation of the mechanisms involved in thrombosis and hemostasis. However, current methods available for determination of FpA by radioimmunoassay (RIA) require rigorous method development. Recently, a commercial kit (Mallinckrodt Corp: M-Kit) for the RIA of FpA has become available which contains all the necessary reagents for the assay. We evaluated this kit and compared it to an assay prepared from a commercial kit (IMCO Corp: I-Kit) which contains only the raw materials. Both assays had similar characteristics and duplicate plasma samples assayed using both methods were not significantly different. Separation of FpA from fibrinogen using bentonite slurry (M-Kit) proved superior to the ethanol precipitation method (I-Kit). The complete kit (M-Kit) will provide the routine hemostasis laboratory with an RIA for FpA which is immediately available.

Key words: Fibrinopeptide A, Radioimmunoassay

INTRODUCTION

Activation of the coagulation system in human blood leads to the generation of thrombin, a proteolytic enzyme responsible for

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the conversion of fibrinogen to fibrin. Thrombin attacks the arginyl-glycine bonds of the Aα chains of the fibrinogen molecule. This interaction leads to the rapid release of 2 moles of fibrinopeptide A (FpA, 16 amino acid residues) followed by the slower release of 2 moles of fibrinopeptide B (FpB) from the Bβ chains (1). The resultant fibrin monomer spontaneously polymerizes to form fibrin. Fibrinogen is a specific substrate for thrombin and hence FpA generation is a direct marker of thrombin formation.

Fibrinopeptide A determinations are of major importance in studies of the ability of anticoagulants to inhibit in vivo thrombin generation. In clinical practice, elevated FpA levels have been reported in thrombotic disorders including disseminated intravascular coagulation (DIC) (2-4), deep vein thrombosis and pulmonary embolism (5-7). Of particular interest are the findings of elevated FpA levels in various clinical disorders where evidence of DIC has not been clearly established such as collagen-vascular disease (4,6) neoplasia (3,5,6) and septicaemia (5,6). Thus, FpA determinations would provide a sensitive index of in vivo thrombin formation both in a clinical and research setting.

Several radioimmunoassays (RIA) have been described for the estimation of FpA concentration beginning with the pioneer work of Nossel et al in 1971 (8).

The major drawback to the widespread use of the FpA assay has been the lack of a commercial source of reagents needed for the assay. The original reported methods for the purification of FpA, raising of the antiserum and subsequent RIA method deve-

lopment was beyond the scope of many laboratories. Because of this, IMCO Corporation (Stockholm Sweden) packaged all the raw materials required for the assay (I-Kit; FpA standard, antisera, and iodination grade FpA). While this allowed a greater dissemination of the assay, interlaboratory comparisons of FpA levels were difficult because of the different assay methodologies adopted. Laboratores not equipped for protein iodination or method development such as most clinical labs, were still left out.

Recently Mallinckrodt Corp. (Chicago Illinois) introduced a complete RIA kit for FpA determinations (M-Kit). This kit, as supplied, provides an FpA assay directly applicable to all laboratories equipped for RIA, particularly important for the clinical application of FpA determinations. This kit would also allow a greater use of the assay for research where FpA determinations are useful but the expense and time required to develop the assay would not be justified.

Because of the wideranging applications of this complete RIA kit for FpA, this project was undertaken to:
1) evaluate and characterize the M-Kit as supplied and 2) compare the M-Kit to an FpA assay developed and based on components obtained from IMCO Corp. The second objective was included to provide a reference point as the I-Kit reagents are the only other commercial source of FpA materials.

MATERIALS

Standard grade FpA, iodination grade FpA (desamintyrosyl-FpA) and rabbit anti-human FpA antisera were purchased as a kit (I-Kit)

from IMCO Corp. (Stockholm Sweden). Additional materials required for the assay were: polyethylene glycol (PEG, MW 6000), chicken egg ovalbumin (Sigma Chemicals, St. Louis Mo. USA) and goat anti-rabbit gammaglobulin (GARGG) (Antibodies Incorp. Davis Ca USA).

The M-Kit was a gift from Mallinckrodt Canada Limited (Mississauga Ont. Canada). The materials supplied in the M-Kit included: ^{125}Iodine-labelled FpA, prediluted FpA standards, rabbit anti-human FpA antiserum, PEG-GARGG solution, bentonite slurry and an anticoagulant consisting of 100 IU heparin, 100 IU aprotinin and 0.74 nmoles EDTA per ml of solution. No additional reagents were reagents were required for the assay.

Blood samples were collected from 20 in-hospital patients in order to obtain a range of FpA values. The blood was collected from an antecubital vein, without stasis, into 10 ml evacuated tubes containing 1.0 ml of anticoagulant supplied in the Mallinckrodt kit. Once the sample was obtained, the tube was placed in an ice-water bath, transported to the laboratory, then centrifuged at 2000x g for 30 minutes at 4°C. Triplicate 1 ml samples were prepared from each specimen and stored deep frozen (-70°C) until processing.

METHODS

A) FpA RIA BASED ON IMCO COMPONENTS

i) Assay Reagents:

The buffer used for this assay consisted of TRIS 50 mmol/L, pH 8.5 with NaCl 100 mmol/L and ovalbumin 10 g/L.

The FpA antiserum wsa diluted 1:2000 with the assay buffer to which 10 IU/mL heparin and 10 g/L of normal rabbit serum were added.

The separating reagent used was an equal mixture of GARGG (diluted 1:20 in assay buffer) and PEG, 10 g/L.

ii) Radioiodination of FpA:

The iodination of desaminotyrosyl-FpA (dFpA) was carried out as described in the IMCO protocol. Ten micrograms of dFpA were reacted with 2 mCi of carrier-free ^{125}Iodine (New England Nuclear, Lachine P.Q. Canada) in the presence of 26.25 ug of chloramine-T in a total reaction volume of 50 uL. The materials were mixed for thirty seconds, then the reaction was terminated by the addition of 75 ug sodium metabisulfate in 25 ul TRIS NaCl buffer, pH 7.5. The iodinated peptide was separated from iodide and peptide degradation products by chromatography on a 0.9 x 60 cm column of Sephadex G-10 in a buffer composed of TRIS 50 mmol/L, pH 7.5, NaCl 100 mmol/L and ovalbumin 1 g/L, at a flow rate of 0.50 ml/min. Fractions of 0.5 ml were collected and a 10 ul aliquot of each was used for radioactive counting. (Figure 1).

The elution pattern obtained using this protocol was similar to that described by Urden (11). Peak 1 is proposed to be aggregated and damaged dFpA and peak 3 is nonreacted iodide. Peak 2, monomeric iodinated dFpA was diluted in 3.5 ml TRIS/NaCl buffer, pH 7.5, to which 80 mg. ovalbumin was added. The iodinated dFpA solution was dispensed in 50 uL aliquots and stored at -70°C until use. The mass of tracer was determined by incubating increasing

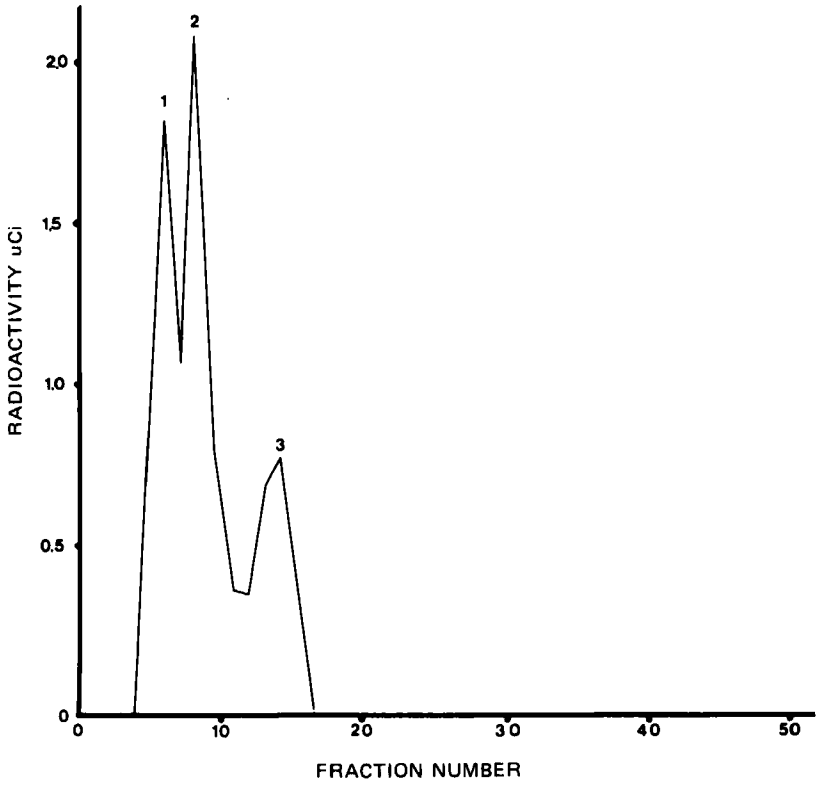

Figure 1: Elution pattern obtained by passing FpA iodination reaction mixture through a 1.0 x 10.0 cm. column packed with Sephadex^{R} G10.

amounts of tracer with a standard curve (10) and in combination with the corresponding counts, the specific activity of the tracer in uCi/ug (usually from 90-150 uCi/jg).

iii) Preparation of Plasma Samples:

Plasma samples were processed to remove fibrinogen using an ethanol precipitation method adopted from the IMCO protocol but without the dialysis step. The protocol involved adding 1.0 mL

ice-cold ethanol (> 99%) to 1.0 mL plasma. After thorough mixing, the sample was kept at -20°C for 30 minutes then centrifuged for 30 minutes at 0°C. The supernatant was then transferred to a clear polypropylene tube and the process repeated two more times. The resultant fibrinogen-free plasma was kept at 0°C until processing.

iv) Radioimmunoassay:

The optimized radioimmunoassay for human FpA using I-Kit reagents was developed and characterized according to the method described by Walker (10). The protocol for performing the FpA-RIA involved the incubation of 100 uL of test sample, standard or buffer with 100 uL of FpA antiserum (diluted to bind 50% of the tracer in the absence of unlabelled FpA) and 50 uL of ^{125}I-dFpA. Because the dialysis step was omitted from the plasma extraction procedure, the concentration of ethanol in the test samples was compensated for in the assay by adding an equal concentration to the standards. Once equilibrium was achieved, the bound and free moeities were separated using a combination of GARGG and PEG. Following centrifugation for 20 minutes at 2000x g at 4°C, the resultant supernatant was aspirated and the remaining pellet (bound fraction) counted in a Beckman 7000 Automatic Gamma Counter.

This assay was determined to come to equilibrium in 18 hours at 4°C and once equilibrium was achieved, to be non-reversible in the presence of excess unlabelled FpA (100 ng). The thermodynamic characteristics of the assay revealed a temperature

dependent system with the greatest equilibrium constant (Ka) at $4^{o}C$ (Ka; $4^{o}C$ - 1.28×10^{9} l/m, $22^{o}C$ - 0.75×10^{9} l/m; $37^{o}C$ - 0.8×10^{9} l/m).

v) Calculations:

Scatchard plots were generated from the assay data with the bound/free values as ordinate expressed as y/1-y where y= bound/total counts and y (L* + La) as the abscissa where L* represents the mass of labelled ligand added to each tube and La is the mass of standard added (9). After corrections were made mathematically for the apparent upper and lower limits of the assay (10), estimates of the binding capacity (x-intercept) and equilibrium constant (Ka, derived from slope of line and converted to molar units) were made.

B) CHACTERIZATION AND EVALUATION OF MALLINCKRODT KIT

In the basic M-Kit radioimmunoassay, 200 ul standard or plasma, 100 ul ^{125}I-FpA and 100 ul FpA antiserum were incubated at room temperature for one to two hours. The bound fraction is then precipitated using a double antibody reagent. The first step in the evaluation of the M-Kit is to determine the optimal incubation time and temperature.

i) Thermodynamic Characteristics:

Three identical standard curves, as supplied in the M-Kit incubated at either $4^{o}C$, $22^{o}C$ or $37^{o}C$ were used to determine the thermodynamics of the assay. The equilibrium constant, Ka was determined from Scatchard plots constructed from the data generated by these standards (10).

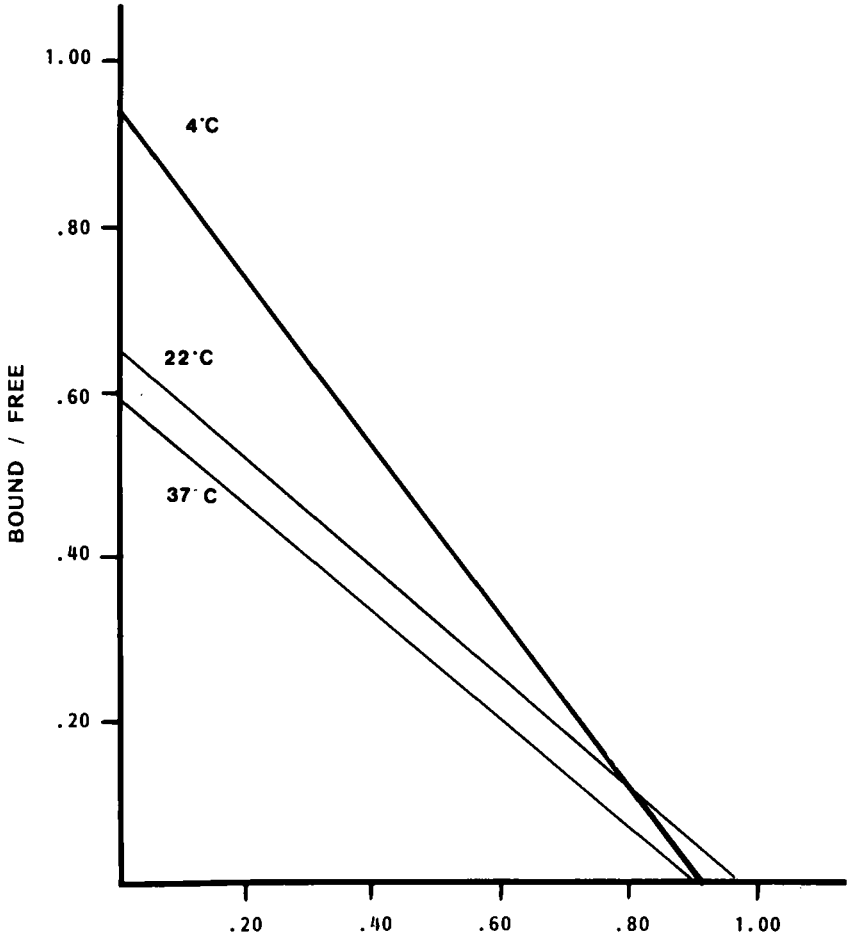

Figure 2: Scatchard plots derived from M-kit standard curves incubated at 4°C, 22°C and 37°C.

ii) Forward Reaction Rate:

The time required for the assay to reach equilibrium at the optimum temperature, was determined by adding the antisera to a series of tubes containing only buffer and tracer at timed intervals from 30 minutes to 24 hours.

iii) Preparation of Plasma Samples:

Plasma samples for FpA determinations were processed before assay to separate free FpA from FpA bound to fibrinogen. In the M-Kit protocol, fibrinogen was removed from plasma using a suspension of bentonite (0.8 mL buffer + 0.2 mL solid). The procedure consisted of adding 1.0 mL of the bentonite slurry to 0.5 mL plasma. After thorough mixing, the bentonite was precipitated by centrifugation at 2000x g for 10 minutes at $4^{o}C$. The resultant supernatant was then transferred to a clear polypropylene tube and stored at $4^{o}C$ (no longer than 24 hours) until processing.

The influence of prolonged exposure of plasma to bentonite was determined by incubating 10 duplicate plasma samples for either 5 minutes, as suggested in the M-Kit protocol, or four hours before centrifugation. Recovery of FpA was also determined in these samples by adding 10,000 cpm of ^{125}I-FpA to each before processing.

iv) Matrix Effects Due to Plasma:

A discrepency may exist in antibody-ligand binding between plasma samples and standards due to the media in which they are suspended. In order to rule out this possibility, a standard curve was incubated in the presence of either extracted plasma or additional buffer.

v) Reproducibility:

The reproducibility of the M-Kit RIA was assessed by preparing samples of FpA standard diluted in assay buffer to a concentration of either 1.5 ng/mL or 5.0 ng/mL. The within assay coefficient of variation was calculated based on the results obtained from 20 low and 20 high samples. The between assay variation was then assessed by repeating this procedure using a fresh kit one month later.

RESULTS

CHARACTERISTICS OF M-KIT RADIOIMMUNOASSAY

i) Effect of Temperature

The M-Kit assay produced similar results to the established I-Kit assay, characteristic of a temperature dependent system (Figure 2). The equilibrium constants derived from the Scatchard plots were: $4^{\circ}C$ - 8.0×10^{-8} L/M, $22^{\circ}C$ - 4.0×10^{-8} L/M and $37^{\circ}C$ - 3.8×10^{-8} L/M.

ii) Forward Reaction Rate:

Because the assay appeared to be temperature dependent, the forward reaction rate at $4^{\circ}C$ was determined. The assay achieved 90% maximum binding by eight hours and had reached equilibrium by 16 hours at $4^{\circ}C$ (Figure 3).

iii) Matrix Effects Due to Plasma

The standard curve to which an extracted plasma had been added was compared to the curve to which buffer had been added. The difference between the calculation result of the plasma standard curve and the buffer standard curve corresponded to the FpA level of the added plasma (Table 1). Matrix effects

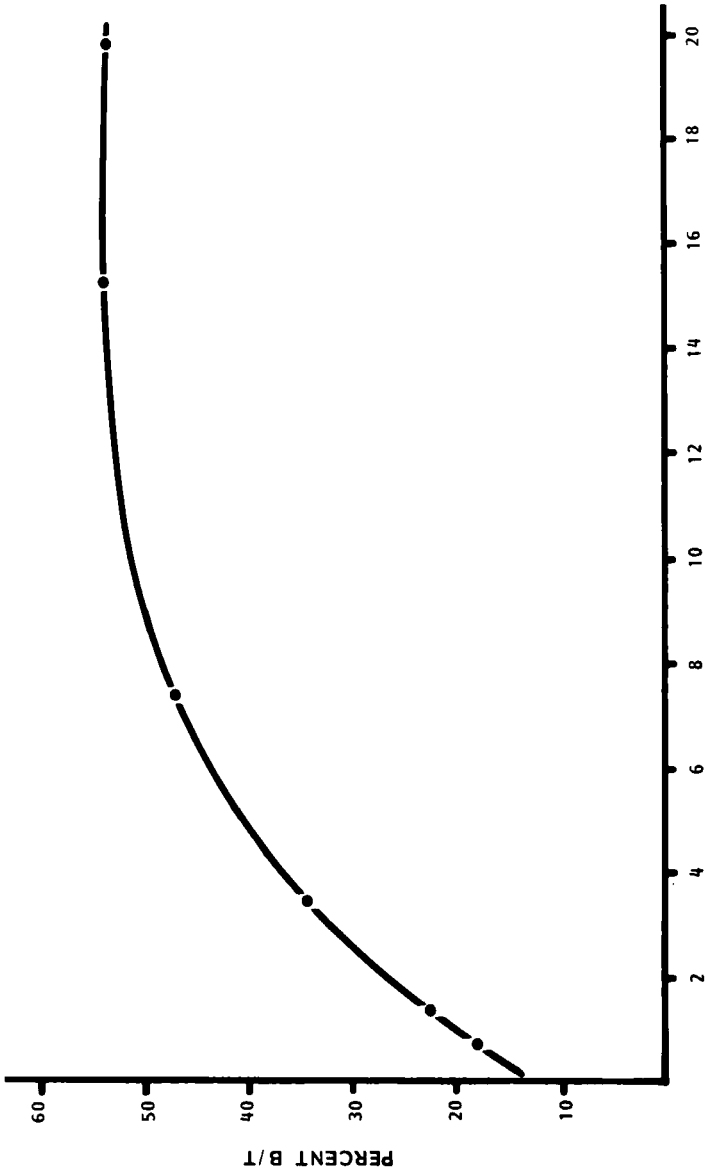

Figure 3: Forward reaction rate of the M-kit assay incubated at 4°C.

TABLE 1.

MATRIX EFFECTS DUE TO PLASMA

Standard Concentration	Calculated Concentration Standard + Plasma	Difference
0.0	12.2	12.2
2.5	14.7	12.2
5.0	17.8	12.8
10.0	24.2	14.2
20.0	32.6	12.6

* Results are expressed in ng/mL.

were routinely monitored over a six month period by assaying samples straight and diluted 1:2. When corrected for dilution, the assay results were consistently within 2 SD of the neat sample.

iv) Reproducibility:

Twenty samples prepared by diluting standard FpA in buffer to a concentration of 1.5 ng/mL and twenty samples at a concentration of 5.0 ng/mL were assayed using the M-Kit, then reassayed one month later using a new M-Kit. The interassay coefficient of variation was 2.5% at 1.5 ng/mL and 6.0% at 5.0 ng/mL. The interassay coefficient of variation was 5% at the lower level and 12% at the higher level.

COMPARISON OF THE M-KIT WITH THE I-KIT

i) Comparison of the Tracers and Antibody:

The M-Kit antibody and tracer were substituted in the assay developed for the I-Kit reagents. Using the protocol developed based on the I-Kit reagents after incubation at room temperature for 2 hours, 52% of the tracer was bound to the antibody (1:6000 final conc.) in the absence of unlabelled ligand. A 10% reduction in binding occurred at 0.70 ng/mL and a 50% inhibition occurred at 6.5 ng/mL. Substitution of the M-Kit antisera into the assay resulted in 50% binding in the absence of unlabelled FpA, a 10% reduction of binding at 0.58 ng/mL and a 50% reduction of binding at 6.0 ng/mL. Substitution of the M-Kit tracer in the assay produced no significant changes from the established I-Kit assay.

ii) Comparison of Assayed Results:

Twenty samples were extracted using the bentonite supplied in the M-Kit and assayed by both the M-Kit assay and the assay developed using the I-Kit. Using standards prepared from the I-Kit, the samples ranged from 0.70 to 19.50 ng/mL. When compared to the results obtained using the M-Kit, no significant differences were observed ($p > 0.25$, Wilcoxon test) Figure 4.

iii) Sample Preparation:

Because the two assays use different methods of removing the fibrinogen from the plasma, these methods were compared. Twenty duplicate samples were extracted by either the ethanol precipitation procedure as suggested in the I-Kit but without

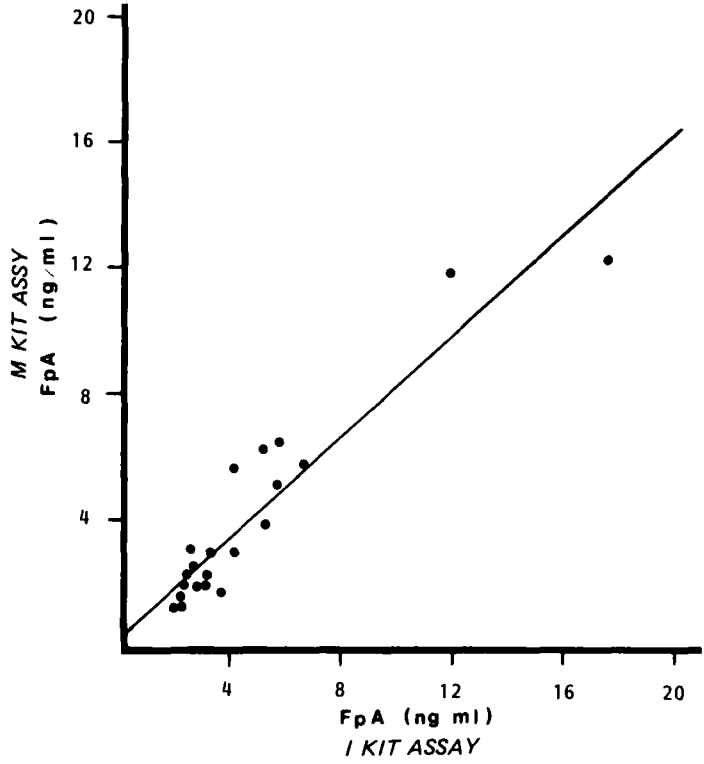

Figure 4: Comparison of the FpA values of extracted plasmas when assayed by either the M-kit or the I-kit method.

dialysis or by using the bentonite slurry as supplied in the M-Kit. The plasma FpA levels in the samples were then compared using the M-Kit assay. There was no significant difference between the assayed results ($p = 0.15$), although there was a suggestion that the results using the bentonite extraction were higher (Figure 5).

Since the ethanol used in extracting the samples might have interfered with the antibody-antigen reaction, the effect of

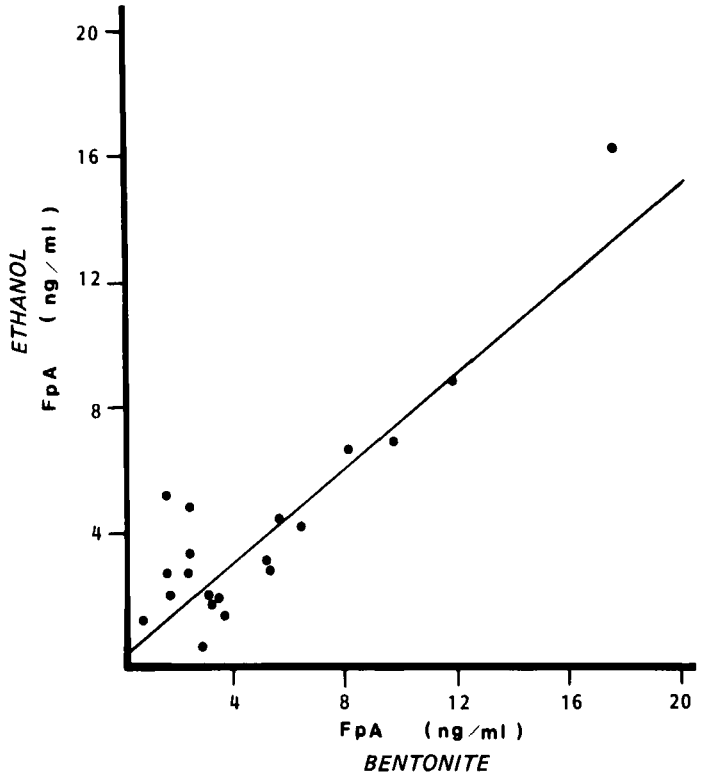

Figure 5: Comparison of the FpA content of test plasmas (I-kit assay) extracted by either the ethanol method or the bentonite method.

ethanol was assessed by incubating a standard curve in the presence or absence of ethanol. It was observed that ethanol present in the sample tended to inhibit the binding of the antibody to the ligand linearly over the range of standards by approximately 6%.

Recovery of ^{125}I-labelled FpA was also determined by both methods using 10 duplicate samples previously assayed for FpA content (FpA range 1.5 - 18.0 ng/mL). In all samples, the

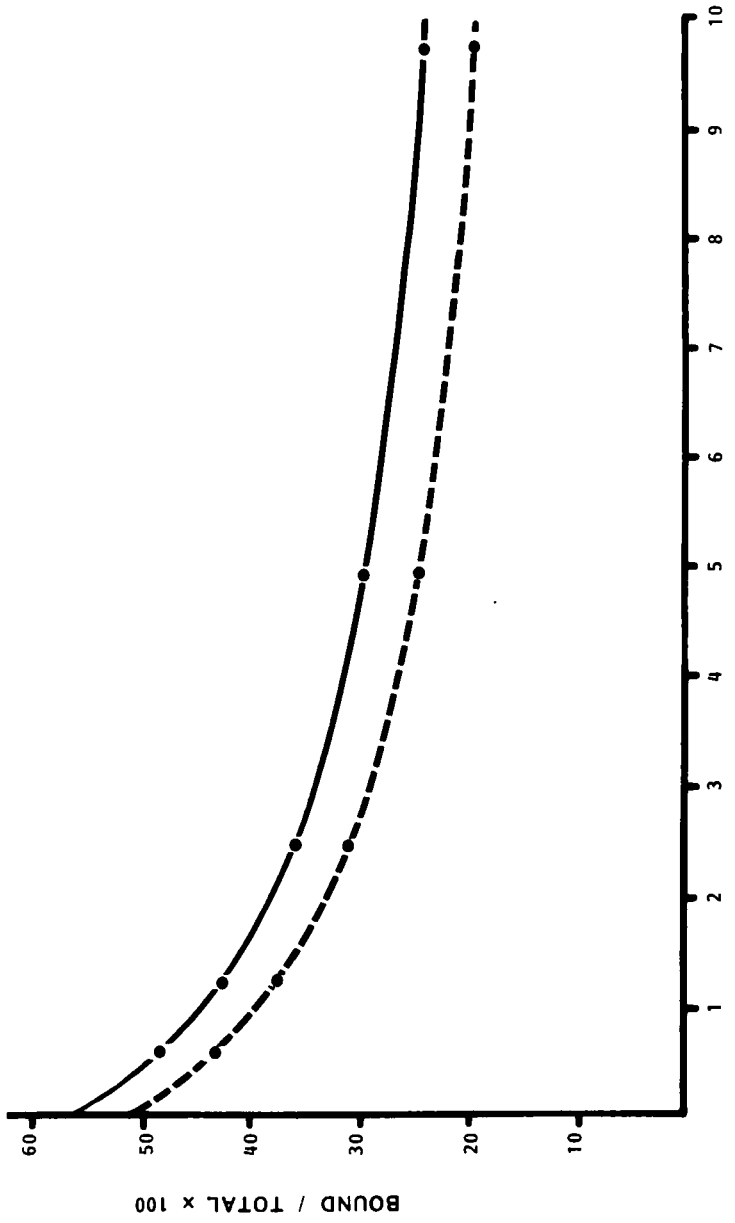

Figure 6: Standard curve incubated in the presence of buffer (———) or 12% ethanol (-----).

TABLE 2.

COMPARISON OF EXTRACTION METHODS

	Ethanol Precipitation	Bentonite Precipitation
Reagent/plasma	1 : 1	2 : 1
Dilution	1 : 2	1 : 2.6
Total time requires	3 hours	15 minutes
Final suspending media	12% ethanol in plasma	plasma and buffer
^{125}IdFpA Recovery	50 + 5%	75 $\pm$ 3%

bentonite method yielded consistently higher recoveries. The mean recovery by the ethanol precipitation method was 50% $\pm$ 5%, whereas the mean recovery by the ethanol precipitation method was 75% $\pm$ 3%.

Table 2 summarizes the findings in the comparison of the two methods. Because prolonged incubation of the plasma in the presence of bentonite may result in absorption of FpA, this potential "stripping effect" of the bentonite was checked by extracting twenty duplicate samples for either 5 minutes, as specified in the M-Kit instruction, or for 4 hours at room temperature. No significant difference was observed in the assayed results of the duplicate samples ($p > 0.25$, Wilcoxon Test).

DISCUSSION

The measurement of FpA by radioimmunoassay is becoming increasingly important in the investigation of disorders of hemostasis and thrombosis (2-6). However, the assay has not been widely applied because of the complexity of developing the assay. Evaluation of the FpA assay in various clinical situations from data generated by various labs has also been complicated by the lack of a standard method for performing and reporting the assay.

This study reports the characterization of a complete radioimmunoassay kit for FpA (M-Kit) suitable for use in routine RIA laboratories and compares this kit to an assay developed using raw materials (I-Kit). The M-Kit assay was characterized as a temperature dependent system, most sensitive at $4^{o}C$. These assay characteristics are similar to those in an assay we developed based on the I-Kit components and to other FpA assays reported in the literature (9,12).

Very sensitive FpA assays requiring delayed addition of tracer were originally developed because of the poor FpA recoveries during sample preparation. Several modifications of the original extraction/dialysis method have been developed including ethanol precipitation without dialysis (12,13) or the use of bentonite (14-16). Because of the greater recovery of FpA with these technically easier methods, it is now possible to use a more rapid, less sensitive assay, which is due to the greater efficacy of the extraction methods. We adopted the ethanol precipitation (without dialysis) because of the low cost of materials and simplicity of the technique. Recovery studies using this method were similar to

those previously published (13,17). However, the disadvantage of this method is the ethanol residue in the sample influences the antigen-antibody binding. In the M-Kit, the bentonite adsorption technique was used. Although this technique is much faster and simpler than other methods, it has been associated with inconsistent results due to variations in bentonite batches (14,15) which will now be resolved by a single commercial source. When we compared samples extracted by bentonite to samples processed by the ethanol precipitation method, the results were not significantly different. However, there tended to be a lot of variation associated with FpA levels below 3 ng/mL. This may be the result of the interference of the ethanol in the sample at the low end of the assay range.

Direct comparison of reagents in both assay systems demonstrated that the various components (tracer, antibody) were freely interchangeable. Hence, the M-Kit assay should provide results comparable to data generated using the I-Kit components.

One important consideration is whether there is a significant difference between the cost of the two assays. Although the cost per sample for materials is less using the I-Kit compared with the M-Kit, additional costs must be considered. Several additional reagents are required for the I-Kit whereas the M-Kit is complete. The I-Kit requires more intensive development and preparation time compared with the M-Kit. Desaminotyrosyl-FpA in the I-Kit deteriorates on storage (18) and may effect the results of the assay.

In a busy developmental RIA lab, where significant numbers of FpA determinations are carried out, the use of the I-Kit reagents should be cost-effective. In a routine laboratory, on the other hand, with limited facilities for assay development, the M-Kit would be most cost-effective, would be immediately available and would not require any additional outlay of development time or supplementary reagents.

ACKNOWLEDGEMENTS

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