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Determination of fibrinogen in plasma by high-performance immunoaffinity chromatography

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ABSTRACT

A high-performance immunoaffinity chromatographic (HPIAC) method for fibrinogen was developed which had several advantages over existing methodologies including increased linear range and no interference from heparin. Several modifications of usual HPIAC procedures were necessary including the employment of a methacrylate polymeric support to reduce non-specific adsorption and the addition of urea to a pH 2.1 elution buffer to affect elution. A significant split-peak effect (*i.e.*, unretained fibrinogen) was noted at higher flow-rates and at higher fibrinogen concentrations, which was shown to be temperature-dependent, with the amount of fibrinogen retained on the column increasing with increased temperature.

INTRODUCTION

Fibrinogen plays a central role in supporting hemostatic plug formation. Below normal plasma fibrinogen levels are seen in disorders such as consumptive coagulopathies, major hemorrhage, fibrinolytic therapy, and hereditary disorders involving abnormal structure (dysfibrinogenemia), decreased levels (hypofibrinogenemia), or totally absent levels of fibrinogen (afibrinogenemia) [1]. Patients will develop an increased risk of bleeding at fibrinogen levels below 0.5 mg/ml (reference range is approximately 1.7–4.0 mg/ml), necessitating corrective therapy [1]. Increased fibrinogen in plasma is seen in conditions that produce an acute inflammatory reaction, such as myocardial infarction and ischemic heart disease [2–5], surgery [6], burns, fractures and other injuries [7]. Increased fibrinogen is associated with an increased risk for cardiovascular disease [8,9] and has been proposed as a prethrombotic marker for atherosclerosis [10,11]. Increased fibrinogen levels have also been used to identify patients at risk for thromboembolism after a myocardial infarction, for which appropriate anticoagulation might reduce morbidity [5]. Neoplastic processes, pregnancy and oral contraceptive use also lead to an increase in fibrinogen levels [12]. An important use of fibrinogen in clinical analysis is in monitoring thrombolytic therapy [13], which is administered to patients exhibiting various thrombotic conditions, including myocardial infarction, peripheral artery occlusion, deep vein thrombosis, and pulmonary embolism [14,15].

Several methods based on different principles currently exist for the measurement of fibrinogen. Included are salt fractionation methods [16], total clottable fibrinogen [17,18], thrombin clotting time [19,20], turbidimetric methods [21,22], prothrombin time derived methods [12], and radial immunodiffusion (RID) [23]. The advantages and disadvantages of some of these methods have been compared [24–27]. Only the thrombin

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clotting time and the RID methods are acceptable with respect to both accuracy and precision. Because the RID method is a cumbersome, time-consuming procedure (taking three days to perform), the thrombin clotting time is more commonly used in the clinical laboratory for the determination of fibrinogen.

There are significant interference problems with both the thrombin clotting time and RID techniques. Fibrinogen degradation products (FDP), which are generated from thrombolytic therapy, and heparin, which is frequently administered in thrombolytic conditions, give significant negative interference in the thrombin clotting time assay [12,20,27–30], while FDP leads to positive interference in the RID method [23]. We seek to address these interference problems by the development of a high-performance immunoaffinity chromatographic (HPIAC) technique. The present work addresses the interference of heparin, while future HPIAC work will address the interference of FDP.

The present work investigates preliminary issues concerning the chromatography of fibrinogen on HPIAC columns, utilizing columns containing polyclonal antibodies to fibrinogen. These issues are the high amount of non-specific adsorption of fibrinogen onto conventional high-performance support materials, immobilization of active antibody onto the support, conditions of dissociation of fibrinogen from the antibody while maintaining the solubility of fibrinogen in the mobile phase, and the kinetics of interaction of fibrinogen with the immunosupport.

EXPERIMENTAL

Reagents

Purified human fibrinogen (used as standards) and polyclonal rabbit anti-human fibrinogen antibodies (immunoglobulin G, IgG) were donated (Dr. John Shainoff, Section on Thrombosis Research, Cleveland Clinic Foundation, Cleveland, OH, USA). High-performance chromatographic support materials used were: Nucleosil 4000-10 silica (10 μm diameter, 4000 Å pore diameter) (Alltech, Deerfield, IL, USA), Hydrazide Avid-Gel P hydrophilic polymer (40–120 μm, 1000 Å) (BioProbe International, Tustin, CA, USA), Affi-Prep 10 and Affi-Prep Hydrazide methacrylate polymers (40 μm, 1000 Å) (Bio-Rad, Hercules, CA, USA). Diol silica was prepared using 3-glycidoxypropyl-trimethoxysilane (Huls Petrarch Systems, Bristol, PA, USA) as given in a published procedure [31]. Nucleosil 4000-10 hydrazide-diol silica was prepared by Dr. David Hage (Department of Chemistry, University of Nebraska-Lincoln, Lincoln, NE, USA). All other chemicals were reagent grade or higher. Distilled, deionized water was used to prepare all solutions and mobile phases.

Apparatus

Immunoaffinity chromatography was performed with a Spectra-Physics HPLC WINner system (Spectra-Physics, San Jose, CA, USA) consisting of an SP8800 ternary pump, an SP8780 autosampler, an SP4270 integrator, and an Epson Equity I+ personal computer. A UVIS 230 spectrophotometric detector (Linear Instruments, Reno, NV, USA) was used. A Model 9500 Isotemp circulator (Fisher Scientific, Pittsburgh, PA, USA) was used to pump water through a HPLC column water jacket (Alltech) to control the column temperature for the temperature studies. HPIAC columns were packed using a HPLC slurry packer (Alltech). Automated fibrinogen thrombin clotting time assays were done on a Dade Diagnostica Stago ST4 (Dade Diagnostics, Aguada, Puerto Rico) at the Cleveland Clinic (Developmental Hematology Section).

Samples

Standards were prepared by diluting a purified fibrinogen stock solution (27.2 mg/ml, >98% pure) in 0.02 *M* sodium phosphate buffer, 0.15 *M* NaCl, pH 7.0 (application buffer). The fibrinogen stock solution was stored in 0.05-ml aliquots at −70°C until use. Blood samples from random patients were collected in tubes containing 3.8% sodium citrate anticoagulant in the proportion of 1:9 anticoagulant to blood at the Cleveland Clinic. Plasma was immediately separated by centrifugation, analyzed for fibrinogen by the Dade Di-

agnostica Stago ST4 and frozen at −70°C prior to analysis by HPIAC. All plasma samples were diluted 1:10 in application buffer prior to chromatography.

Procedures

Preparation of immunosupport. The high-performance immunoaffinity support was prepared by immobilization of anti-human fibrinogen antibodies to the Affi-Prep Hydrazide methacrylate polymer support. The reaction conditions for immobilization of antibody onto the hydrazide support are described below. All reactions were carried out with shaking using a wrist-action shaker (Burrell, Pittsburgh, PA, USA). All filtering and washing of the packing material was accomplished with suction using a medium-porosity glass filter. Polyclonal anti-human fibrinogen immunoglobulin (4.24 mg/ml, 400 μl) was dialyzed using Spectra/Pore 7 dialysis tubing, molecular weight cut-off 25 000 (Fisher Scientific) against three 1-l volumes of 0.02 *M* sodium acetate, 0.15 *M* NaCl pH 5.0 buffer at 4°C over a period of 48 h, to remove components present in the antibody solution that may interfere with the immobilization reactions. After dialysis, sodium periodate was added to the antibody solution to achieve a final concentration of 10 m*M* and this mixture was reacted for 30 min at room temperature in the dark. Glycerol (50 μl) was added to the reaction mixture and shaken for 10 min to stop the oxidation reaction. The oxidized IgG solution was then dialyzed against three 1-l volumes of coupling buffer (0.02 *M* sodium acetate, 0.15 *M* sodium chloride, pH 4.5) over a 48-h period at 4°C. A 1-ml quantity of the Affi-Prep Hydrazide support was washed successively with 20 ml of distilled, deionized water and with 20 ml of coupling buffer. The dialyzed antibody solution was added to the washed support and reacted for 30 h at 4°C.

The amount of antibody coupled to the support was determined by quantitating the concentration difference of antibody in the supernatant before and after coupling, based on absorbance at 280 nm (supernatant from the reaction mixture along with two 3-ml coupling buffer washings of the packing material were combined). The amount of antibody immobilized per volume of support was 1.2 mg/ml.

Column packing procedure. Chromatographic studies were done not only on the Affi-Prep Hydrazide support, but initially on other chromatographic support materials, to find the support which exhibited the least non-specific adsorption characteristics. The polymeric and silica support materials were packed at 35 and 210 bar, respectively, using application buffer. All columns were 5.00 cm × 0.25 in. O.D. × 4.1 mm I.D. The immunocolumn was stored in 0.02 *M* sodium phosphate, 0.02% sodium azide pH 7.0 at 4°C when not in use.

Chromatographic conditions on the HPIAC column. Chromatographic mobile phases consisted of an application buffer (described previously) and an elution buffer of 0.02 *M* sodium phosphate, 0.15 *M* NaCl, 4.0 *M* urea pH 2.1. The flow-rate was 0.5 ml/min and the column was maintained at ambient temperature. Sample injection volume was 20 μl and the wavelength of detection was 280 nm. Total chromatographic time was 15 min as follows: sample was injected into application buffer at 0 min; a mobile phase change to 100% elution buffer was made at 0.3 min and pumped until 5 min; then a switch was made back to application buffer until 15 min, to reequilibrate the column. The time for a programmed solvent change to reach the detector at a flow-rate of 0.5 ml/min was 4.3 min.

Characterization studies. For all studies given below 20-μl samples were injected into application buffer (except for the pH non-specific adsorption studies), pumped at 0.5 ml/min (except for the flow-rate studies) at ambient temperatures (except for the temperature studies). Non-specific adsorption of fibrinogen to different support materials was determined by making multiple injections of a fibrinogen standard (2 mg/ml) onto columns of the different support materials. After conditioning a column with ten injections, one final injection was made and the percentage of fibrinogen retained was calculated for this injection [peak areas were compared for column and blank tubing (no column in place)]. A study of

the effect of pH on the non-specific adsorption of fibrinogen on the Affi-Prep Hydrazide was undertaken using mobile phases consisting of pH 7.0 and pH 6.0 0.02 *M* sodium phosphate buffers (no NaCl) and pH 2.1 0.02 *M* sodium phosphate buffer with 4.0 *M* urea (no NaCl). Analytical recovery was determined by measuring elution peak areas (peak 2 in Fig. 1C) of ten fibrinogen standards (0.1 to 3.4 mg/ml) and comparing them with peak areas obtained from injecting the same standards on blank tubing (no column present). Linearity was determined by injection of fibrinogen standards (0.1–3.4 mg/ml). The effect of flow-rate on the chromatography of fibrinogen was determined by injecting a 0.6 mg/ml fibrinogen standard onto the HPIAC column at flow-rates of 0.25–2.0 ml/min. Temperature studies were performed by injecting a 1.0 mg/ml fibrinogen standard onto the HPIAC column at temperatures ranging from 4 to 37°C.

RESULTS AND DISCUSSION

Chromatography

Fig. 1A, B and C show chromatograms of a plasma sample, a blank and a baseline-corrected plasma sample, respectively, obtained by chromatography on the anti-fibrinogen HPIAC column. The first peak in Fig. 1A and C is the non-retained peak, consisting of all the constituents not retained by the column that absorb at 280 nm. The second peak is the fibrinogen peak, which eluted from the column upon changing the mobile phase from the pH 7.0 buffer to the pH 2.1 buffer with 4 *M* urea. Urea was an essential component of the elution buffer, being necessary for solubilization of fibrinogen at low pH. The throughput time for this chromatographic set-up was 15 min, which included a 10-min time of equilibration in switching from elution to application buffer. The chromatographic set-up, however, was not optimized with respect to throughput time. The use of minicolumns in HPIAC has been shown to reduce analysis time to less than 1 min [32] and thus a throughput time of a couple of minutes is conceivable for this method.

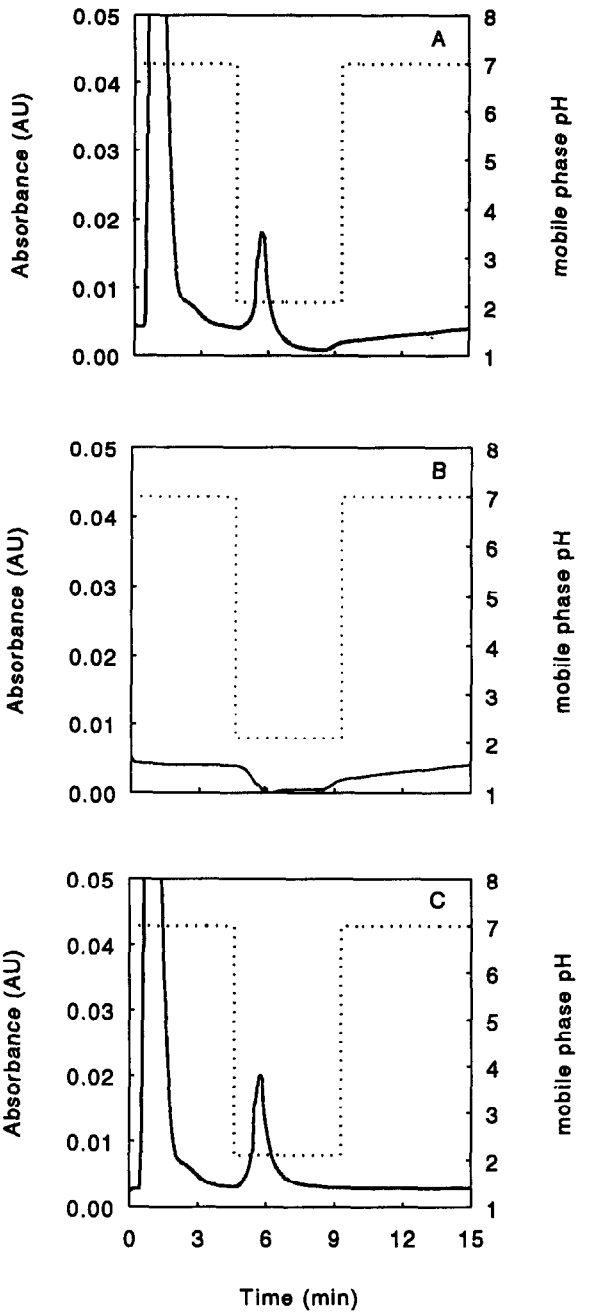

Fig. 1. Chromatography on anti-fibrinogen HPIAC column of (A) 20 μl of a 1:10 dilution of patient plasma sample (3.43 mg/ml fibrinogen), (B) blank (no sample injected), and (C) baseline-corrected 1:10 diluted plasma sample. Application buffer was 0.02 *M* sodium phosphate, 0.15 *M* NaCl pH 7.0. Elution buffer was 0.02 *M* sodium phosphate, 0.15 *M* NaCl, 4.0 *M* urea pH 2.1. Mobile phase gradient, as it appears at the detector, is given by dotted lines. Peak 1 (lowest retention time) is non-retained components and peak 2 is eluted fibrinogen.

Support characteristics

A methacrylate polymer support was used in this work. It was chosen after a comparative study of non-specific adsorption of fibrinogen onto various high-performance affinity chromatographic supports. The results are summarized in Table I. Although the N-hydroxysuccinimide methacrylate support has the lowest non-specific adsorption (9% of injected fibrinogen), the hydrazide-activated methacrylate polymer (showing 22% of the fibrinogen non-specifically adsorbed) was chosen because of its reaction chemistry. The hydrazide group on the support allows for site-directed attachment of the antibody to the support via reaction of carbohydrate groups (located exclusively on the antibody's Fc region and oxidized by periodate) to the hydrazide group to form hydrazone linkages [33,34]. This technique has been shown to enhance binding capacity of immobilized antibodies for antigens over amine group attachment methods, which generally have only a 1–30% yield of active immobilized antibodies [33]. The hydrazide methacrylate polymer has a pore diameter of 1000 Å, which is a minimum requirement, knowing that the dimensions of fibrinogen are 460 × 90 Å [35] and considering that the pore diameter may be diminished by two antibody lengths (the dimensions of IgG are 187 × 82 Å [36]). The methacrylate support can withstand pressures up to 69 bar.

From the non-specific adsorption data given in Table I, it can be seen that the hydrazide functionality on both silica- and polymeric-based supports increased the amount of non-specific adsorption. This non-specific adsorption was shown to be pH-dependent. Experiments were done comparing the non-specific adsorption of fibrinogen on the Affi-Prep Hydrazide support using mobile phases consisting of pH 7.0 and pH 6.0 0.02 *M* sodium phosphate buffers. The non-specific adsorption was found to be 32 and 65%, respectively. This increased non-specific adsorption seen with decreased pH results presumably from the increased protonation of the hydrazide groups at lower pH (the pK_a of hydrazine is 8.2 [37]; the pK_a of the methacrylate hydrazide is expected to be less than this, because of the bonding of hydrazine to a carbonyl carbon on the support), leading to retention of the negatively charged fibrinogen (pI = 5.8 [38]). Non-specific adsorption was decreased to 11% for 0.02 *M* sodium phosphate, 4.0 *M* urea pH 2.1 buffer, as both hydrazide and fibrinogen are positively charged at this pH. The addition of 0.15 *M* NaCl to the pH 7.0 buffer decreased the non-specific adsorption from 32 to 22%. Although further reduction of non-specific adsorption is desirable, the performance of the method was not adversely affected by this amount of non-specific adsorption.

TABLE I

PERCENTAGE OF INJECTED FIBRINOGEN NON-SPECIFICALLY ADSORBED ONTO VARIOUS CHROMATOGRAPHIC SUPPORTS

Support	Percentage of injected fibrinogen
Silica-based	
Nucleosil 4000-10 Diol[a]	71
Hydrazide Nucleosil 4000-10 Diol[a]	100
Polymer-based	
Hydrazide AvidGel P[b]	100
N-Hydroxysuccinimide methacrylate[c]	9
Hydrazide methacrylate[c]	22

[a] Prepared from Macherey-Negel Nucleosil 4000-10.
[b] From BioProbe.
[c] From Bio-Rad.

Split peaks

It was noted in the injection of fibrinogen standards (except for low concentration standards) that a fraction of the injected fibrinogen passed through the column unretained. Clearly this effect was not caused by overloading the column capacity, as seen above. Rather it was due to the slow kinetics of interaction of the analyte with the support material, an effect known as the split-peak effect [39–41]. The equation describing this effect is [39,41]:

$$\frac{-1}{\ln f} = F\left(\frac{1}{k_1 V_e} + \frac{1}{k_3 m_L}\right) \quad (1)$$

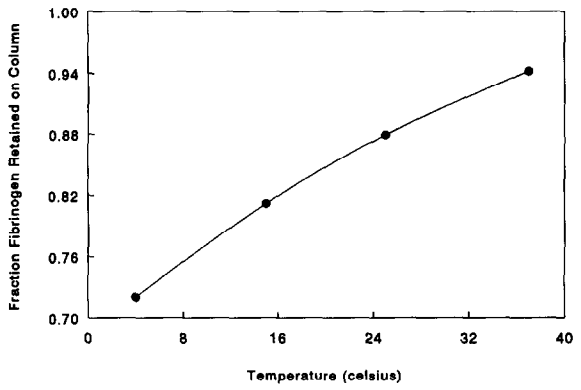

Fig. 2. Effect of temperature on the fraction of injected fibrinogen standard (20 μl of 1.00 mg/ml) retained by the HPIAC column.

where f is the fraction of analyte not retained by the column, F is the flow-rate, m_L is the amount of active immobilized ligand in the column, k_1 is the first-order rate constant for the mass transfer of the analyte from the moving mobile phase to the pores, k_3 is the second-order adsorption rate constant between the immobilized ligand and analyte and V_e is the excluded volume of the column. It is seen from eqn. 1 that there is an increase in the unretained fraction with increased flow-rate. Another factor not accounted for in eqn. 1 (since eqn. 1 applies only to linear elution conditions) is the amount of analyte injected, as an increase in amount of analyte injected (non-linear amounts) causes an increase in f [42]. Both these effects were seen in the present work. In the flow-rate studies f was 0.004 for 0.25 ml/min, 0.019 for 0.50 ml/min, 0.125 for 1.0 ml/min and 0.298 for 2.0 ml/min, when a 12-μg fibrinogen standard was injected. In studies where the amount of fibrinogen injected was varied (flow-rate was 0.5 ml/min), f was negligible for 2–10 μg, 0.053 for 17 μg, 0.102 for 34 μg and 0.208 for 68 μg of fibrinogen. From the above results it was determined that a flow-rate of 0.5 ml/min was acceptable, since the amount of fibrinogen contained in 20 μl of normal plasma sample diluted 1:10 would range from 3.4 to 8.0 μg (reference range is 1.7–4.0 mg/ml).

In addition to the parameters discussed above, work in our laboratory revealed the split-peak effect to be temperature-dependent. Fig. 2 shows the effect of changing column temperature on the fraction retained ($1 - f$). The fraction retained was shown to increase as the column temperature increased from 4 to 37°C. This effect was most likely due to an increase in k_1 and/or k_3 with increased temperature, and is currently under investigation in our laboratory.

Characterization studies

Linearity. Fig. 3 is a calibration plot obtained by the HPIAC method for fibrinogen. A slight curvature is noted in the calibration plot, which is most likely due to the split-peak effect. The linear range was taken to be the range of the first five standards used in this study, from 0.1 to 1.7 mg/ml. The linear range for patient samples (which were diluted ten-fold) would be 1.0–17 mg/ml. Plasma samples with below normal to highly elevated fibrinogen concentrations will fall within the linear range of the assay since the normal plasma fibrinogen concentration is 1.7–4.0 mg/ml, which can increase three- to four-fold in acute inflammatory reaction [42]. This linear range exceeded the range of the thrombin clotting time assay by more than three-fold.

Analytical recovery, reproducibility, and column lifetime. Recovery was determined by comparing

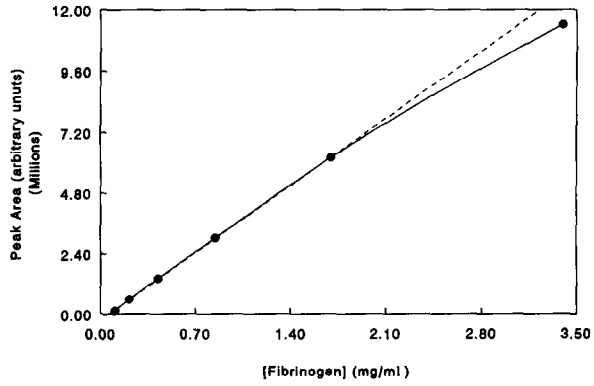

Fig. 3. Calibration plot for 20 μl of fibrinogen standards injected onto the HPIAC column. Solid line is spline fit through all six data points. Dashed line is linear regression fit of first five data points (linear region). Least-squares regression parameters of dashed line are: slope = $3.79 \cdot 10^6$; y-intercept = $-2.18 \cdot 10^5$; r = 1.000.

peak areas for injections on blank tubing with eluted peak areas (peak 2 in Fig. 1C) from injections on the column. Recovery was acceptable, averaging 80.9% (s = 5.6) for ten fibrinogen standards ranging in concentration from 0.1 to 3.4 mg/ml. Within-day reproducibility was determined by making ten consecutive injections of fibrinogen standard (3.0 mg/ml). Precision was very good (C.V. = 1.55%). With respect to column lifetime, a slight decrease in active binding was noted over a six-month period. During this time there were 200 application elution buffer cycles run through the column, with the percentage of non-retained fibrinogen (0.025 mg fibrinogen standard injected) increasing from 8.7 to 15.6% from the beginning to the end of this six-month period.

Interferences. The HPIAC method for fibrinogen was not subject to interference from heparin or other plasma constituents. Because serum is essentially plasma devoid of fibrinogen, all possible interferences in plasma (with the exception of degradation products of fibrinogen and fibrin) could effectively be tested by doing chromatography of serum samples. Injections of serum and blank samples onto the HPIAC support showed no difference in elution peak areas, indicating the absence of interfering components. In addition, bovine serum albumin (20 μl, 5 mg/ml) was injected onto the HPIAC column and greater than 99% passed through the column unretained. Comparison of heparin-spiked fibrinogen standards with unspiked standards showed less than 1% difference in elution peak area at heparin concentrations of 1000 U/ml (which is much greater than the therapeutic range, which is 0.2–0.5 U/ml [1]).

The polyclonal anti-human fibrinogen antibody used in this study is anticipated to cross-react with FDP. For this reason, high levels of FDP in plasma are expected to have a positive bias on fibrinogen values obtained by this method. Use of a monoclonal antibody which does not cross-react with FDP is currently under investigation in our laboratory to deal with this problem.

Patient samples. Twenty patient plasma samples were determined by both a thrombin clotting time method and the HPIAC method. Fibrinogen concentrations for patient samples ranged from 2.56 to 8.78 mg/ml (as determined by the thrombin clotting time method). All samples analyzed by the HPIAC method were diluted 1:10 in application buffer before chromatography. Fig. 4 shows the correlation between patient sample values obtained by the thrombin clotting time method and the HPIAC method (r = 0.954). It should be noted that the value for the fibrinogen stock standard used to prepare the HPIAC calibration standards was determined by the thrombin clotting time technique. Thus, equivalent calibrations were essentially done for both methods. A reasonable slope (0.87) and intercept (0.65) are seen for the comparison plot in Fig. 4.

CONCLUSIONS

Because fibrinogen is essential in monitoring cardiovascular disease, in terms of risk, diagnosis, assessment, and regulation of fibrinolytic therapy, its determination is more and more frequently requested in the clinical laboratory. Reasonable correlation between the thrombin time assay and the HPIAC method was demonstrated in this work, although further characterization is necessary. The HPIAC method exhibited distinct

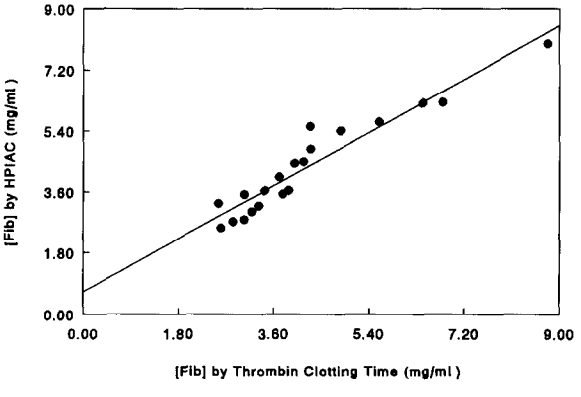

Fig. 4. Methods comparison plot for patient plasma samples (n = 20) comparing HPIAC and thrombin clotting time methods. Least-squares regression parameters are: slope = 0.872; y-intercept = 0.648; r = 0.954.

advantages over the thrombin clotting time assay, not being subject to heparin interference and having increased linear range. The method had a throughput time of 15 min per sample and no sample preparation (besides dilution) was required. Significant advances in HPIAC of fibrinogen reported in this study include the use of methacrylate polymers to minimize non-specific adsorption and the elution strategy of low pH, for the disruption of the antibody-fibrinogen complex, and 4 *M* urea, to solubilize the fibrinogen at low pH. In addition, a discovery applicable to all HPIAC techniques is reported, being the temperature dependence of the split-peak effect. The finding of decreased non-retained analyte fraction with increased temperature implies an important practical application of operating HPIAC columns at elevated temperatures, in cases where split-peak behavior is noted.

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