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Simultaneous Diagnosis of Coagulation Factor XIIIA (F13A) and Plasminogen (PLG) Phenotypes by Polyacrylamide Gel Isoelectric Focusing

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ABSTRACT: In this paper, we report a simple rapid method for simultaneous determination of Coagulation Factor XIIIA (F13A) and plasminogen (PLG) phenotypes by PAGIF with a nominal pH range of 3.5 to 10, followed by immunofixation and silver stain. Critical considerations concerning the conditions of molecular separation and detection strategies are also presented.

KEYWORDS: pathology and biology, genetic typing, isoelectric focusing, Coagulation Factor XIIIA, plasminogen, simultaneous determination, genetic markers, F13A, PLG

The increasing number of electrophoretic genetic markers has emphasized the need to develop simultaneous determination techniques for a variety of purposes, including genetic screening in human genetic studies, biological paternity testing, and forensic science analysis.

The application of isoelectric focusing as a method for molecular separation is particularly useful since the proteins remain immobilized (focused) in their respective isoelectric points. This aspect is considerably relevant for the simultaneous analysis of genetic markers provided that the corresponding isoelectric points are defined at intervals sufficiently separate and therefore easily distinguished, without causing problems of pattern interpretation.

Factor XIII is a proenzyme which, after activation by trombin, forms γ-glutamyl-ε-lysine cross-links between fibrin molecules. The genetic polymorphism of the subunit FXIII A (F13A) was initially described by Board [*1*] along with the existence of two common alleles (F13A*1 and F13A*2) by agarose gel electrophoresis. Plasminogen (PLG) is a circulating plasma protein, the inactive precursor of protease plasmin. The plasminogen-plasmin system plays an important role in the fibrinolitic mechanism. Plasminogen polymorphism was independently reported by Hobart [*2*] and Raum et al. [*3*], who have demonstrated the existence of two common, codominant autosomal alleles.

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In this paper, we describe a method based on isoelectric focusing (IEF) in combination with immunofixation as a strategy for specific detection which enables simultaneous phenotype characterization of both polymorphic systems, PLG and F13A, in order to provide a single, reliable, and reproducable protocol for genetic screening studies.

Materials and Methods

Samples

Blood samples were obtained from healthy individuals and treated with ethylenediaminetetraacetate-disodium (EDTA-NA_2) (10%, w/v) (0.5-mL/mL blood) as anticoagulant. After being centrifuged at 2500 rpm for 10 min the plasma was collected and stored at −20°C for a period not exceeding three years before analysis. Neuraminidase treatment was carried out after incubating 30-μL plasma sample with 30-μL Neuraminidase Type VIII (Sigma, St. Louis) (2.5 U/500 μL) for 2 h at 37°C.

Isoelectric Focusing

Isoelectric focusing was carried out in 0.5- by 230- by 115-mm polyacrylamide gels at a concentration of $T = 5$ and a cross-linking of $C = 3$. Ampholine® (Pharmacia-LKB, Uppsala, Sweden) pH 3.5 to 10 was incorporated with a final concentration of 2% (w/v). Photochemical polymerization was performed under 360-nm light for 25 min using Riboflavin at 0.000 75% (w/v). H_3PO_4 1*M* and NaOH 1*N* were used as anolyte and catholyte, respectively. Native and neuraminidase-treated samples were applied by soaking small pieces of 0.8- by 0.5-cm Whatman No. 1 paper at approximately 2 cm from the anodal strip. Electrical running was conducted at 8°C on a 2117 Multiphor II Unit and a Macrodrive 2997 power supply (LKB, Bromma, Sweden) at the following settings: 1200 V, 20 mA, and 8 W. The pieces of paper were removed after 45 min and focusing was carried out for a further 45 min.

Immunofixation and Silver Stain

Specific antisera, anti-F13A (Behringwerke, Marburg, Germany), and anti-plasminogen (Dako Immunoglobulins, Denmark) were diluted in physiological saline 1:3 and 1:4 (v/v), respectively, and evenly spread on the gel surface within the area comprised between 5.1 and 5.5 cm from the cathode for F13A detection and 3.5 to 4.9 cm for PLG.

The gel was incubated at 37°C in a moist chamber for 1.5 h and then washed overnight in physiological saline to remove the soluble proteins. Excess of NaCl was eliminated after three consecutive 15-min baths in distilled water.

Simplified silver staining was performed according to Budowle and Scott [*4*] with the following minor modifications: potassium dichromate was extensively washed for 7 min minimum using milli-Q water. Treatment with silver nitrate (NO_3Ag) was increased to 0.2% (w/v) for 10 min, and formaldehyde concentration was 1.66% (w/v) in the reduction step.

Results and Discussion

The results of the method described in this paper using untreated plasma samples are shown in Fig. 1. PLG appears as a multiple-banded pattern in the region comprising between 3.5 to 4.9 cm from the cathode, in which the cathodic bands (indicated by white dots) are of special interest in the interpretation of phenotypes [*3*,*5*]. Although homozygotes PLG AA are easily identified, discrimination between heterozygotes PLG AB

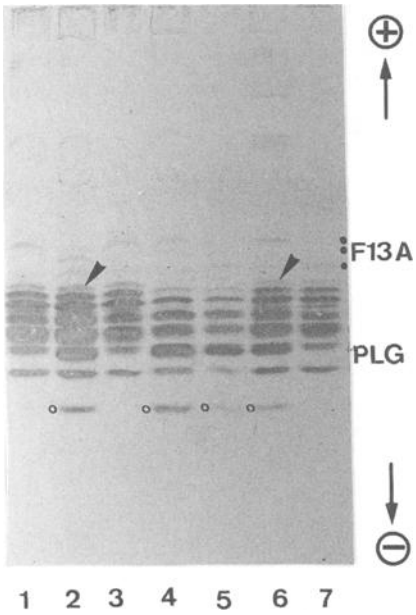

FIG. 1—*F13A and PLG phenotypes by IEF (pH 3.5 to 10) of native plasma samples. F13A: 1, 3, 4, 6 (F13A 1-1); 2 (F13A 2-1); 5, 7 (F13A 2-2). PLG: 1, 3, 7 (PLG AA); 2, 6 (PLG AB); 4, 5 (PLG BB).*

and homozygotes PLG BB may give rise to problems in pattern interpretation. The most anodic bands of the pattern (indicated by arrows) are clearly less marked or even almost absent in the phenotype PLG BB. Regarding F13A, under these conditions their interpretation does not offer any problems; homozygotes F13A 1-1 and F13A 2-2 are shown as a single anodic or cathodic band, respectively. Heterozygotes F13A 2-1 appear as almost symmetrical triplets, but in fact the intermediate heterodimeric band is slightly closer to the most acidic band. Occasionally fainter minor bands may also appear anodally, indicating molecular degradation during prolonged storage [*6*]. The different isoelectric point (pI) intervals at which the bands of both systems focus, facilitate easy discrimination between the systems, the single band corresponds to the F13A 2-2 homozygotes which are clearly separate from the PLG most acidic bands.

Neuraminidase pretreatment of the samples facilitates distinction of PLG phenotypes, as can be observed in Fig. 2. Three reading zones (referred to as I, II, and III, Fig. 2) serve to check the interpretation of molecular phenotypes. The latter zone corresponds to a set of "hole spots" which may be indicative of desequilibrium between antigen and antibody concentrations in this region.

Neuraminidase concentrations seem to have a minimal influence on the reliability of PLG diagnosis, but this is not so with the type of neuraminidase. Sachs and Gützkow [*7*] observed that excellent results are obtained when neuraminidase from *Clostridium perfringens* is used. Concerning F13A, neuraminidase treatment of samples may give rise to additional anodic bands, as indicated by arrows in Fig. 2, and are different from the bands of molecular degradation referred to elsewhere. These bands overlap the bands corresponding to the allele F13A*1 because both have almost the same isoelectric point. Varying neuraminidase concentrations and incubation times do not seem to prevent the apparition of these additional bands. The presence of such bands should be taken into

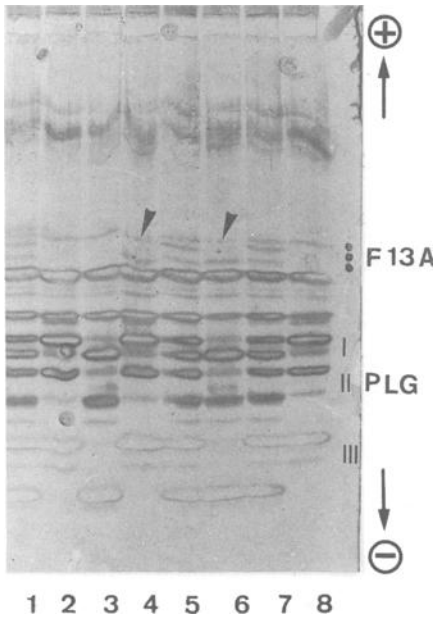

FIG. 2—*F13A and PLG phenotypes by IEF (pH 3.5 to 10) of desialized plasma samples. F13A: 2, 3, 8 (F13A 1-1); 1, 5, 7 (F13A 2-1); 4, 6 (F13A 2-2). PLG: 2, 4, 8 (PLG AA); 1, 5, 7 (PLG AB); 3, 6 (PLG BB).*

account in order to obtain a correct interpretation of the phenotype F13A 2-2, which under no circumstances should be confused with the heterozygotes F13A 2-1. Two criteria aid in distinguishing these two phenotypes: first, the most marked bands correspond to the most cathodic band of the complex (corresponding to the allelic product F13A*2); second, and most important, the homozygotes F13A 2-2 lack the intermediate heterodimeric band, a characteristic of heterozygote subjects.

IEF is the technique of choice when dealing with simultaneous determination of F13A and PLG phenotypes, although agarose thin layer electrophoresis was previously used for individual characterization giving satisfactory results [*8,9*]. Any ampholyte pH range which covers the isoelectric point of these markers can potentially be used. A wide pH range (pH 3.5 to 10) shows that F13A and PLG bandings are clearly separate. Narrower pH ranges (e.g., pH 5 to 7) may also be used, but the resolution obtained is only marginally improved. Notwithstanding, wide pH ranges (pH 3.5 to 10) are preferable for two main reasons: bands are distributed in a closer area, which means that a smaller quantity of antiserum is required for immunofixation, and the time required for an adequate focusing of bands is substantially reduced.

Special attention should be paid to the point of application of the sample. Although this is not critical in the case of anodic or cathodic application for F13A, in the case of PLG the only position is near the anodal edge, as cathodal application leads to blurred and illegible patterns.

Methods based on functional detection, such as fibrinolytic assays for PLG [*2*] and transglutaminase activity for F13A [*1*], have been developed for specific visualization of the above systems. Although both methods give excellent results, unfortunately their application on the same plate with a view to simultaneous PLG and F13A detection is seriously limited for obvious reasons.

In our opinion, immunofixation proves to be an appropriate strategy in these circumstances, given that the antigen molecules are immobilized in the gel matrix as they are progressively linked to the antibodies, thus avoiding molecular diffusion. IEF in combination with immunoblotting is an effective method for phenotyping F13A [*10–12*] and PLG [*13*]. Notwithstanding, conventional immunofixation offers an important advantage: the methodology is substantially simpler and faster than enzymatic immunoassays. In polyacrylamide gels the high sensitivity of silver stain [*14*] enables detection even with minimal amounts of antigen-antibody complexes. This is of special relevance in those circumstances in which the quantity of biological samples is critical. Also, the dramatically reduced volume of antiserum enables considerable savings of expensive reagents.

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