TECHNICAL NOTE

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Group Specific Component Subtyping in Bloodstains by Separator Isoelectric Focusing in Micro-Ultrathin Polyacrylamide Gels Followed by Immunoblotting

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ABSTRACT: The identification of group specific component (Gc) subtypes derived from bloodstains by separator isoelectric focusing in micro-ultrathin polyacrylamide gels (interelectrode distance: 50 mm) containing 4.5 to 5.4 pharmalytes is described. The separation achieved between Gc 1F and Gc 1S bands is compared favorably with that obtained using separator isoelectric focusing in conventional polyacrylamide gels dimensions (interelectrode distance: 110 to 120 mm). The technique is rapid and economical, and the immunoblotting method described is more sensitive than immunofixation followed by silver staining.

KEYWORDS: pathology and biology, group specific component, genetic typing, isoelectric focusing, bloodstains, micro-ultrathin polyacrylamide gel, immunoblotting

Group specific component (Gc) is a serum α_2-globulin, involved with vitamin D-3 transport [*1*].

The Gc protein shows genetic polymorphism. Three common phenotypes Gc 1, Gc 2-1, and Gc 2 were first detected by conventional electrophoresis. The application of isoelectric focusing (IEF) in polyacrylamide gel to the separation of the Gc subtypes by Constant and Viau [*2*] demonstrated that the Gc 1 allele can be subtyped into the Gc 1F and Gc 1S alleles. This allows six common phenotypes, 1F, 1S, 1F 1S, 2 1F, 2 1S, and 2 to be identified.

The Gc subtyping in bloodstains by IEF was first described by Baxter et al. [*3*] using ultrathin layer polyacrylamide gel containing pH 4 to 6 carrier ampholytes. However, by this method the Gc 1 band could not be separated into the Gc 1F and Gc 1S bands. The addition of the separator 3-(morpholino) propanesulphonic acid (MOPS) to ultrathin polyacrylamide gels containing pH 4 to 6 carrier ampholytes allowed the Gc 2, Gc 1F, and Gc 1S bands to separate in bloodstains [*4*].

Following the development by Pharmacia of narrow pH range pharmalytes covering the range of pH 4.5 to 5.4 Westwood [*5*] has reported their use in combination with the addition of the separator *N*-2-hydroxyethyl-piperazine-*N'*2-ethanesulphonic acid (HEPES) as a successful method to separate the Gc subtypes from bloodstains.

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The use of immobilized pH gradients (IPGs) for the separation of Gc subtypes in bloodstains has also been reported [*6*] as the technique with the greatest degree of separation but with less sensitivity than ultrathin polyacrylamide gels containing pH 4.5 to 5.4 pharmalytes.

More recently, Edwards [*7*] reported a method for separating Gc subtypes in bloodstains using ultrathin polyacrylamide gels containing a mixture of the separators HEPES and MOPS and pH 4.5 to 5.4 pharmalytes. The separation achieved by this method is comparable with the separation observed using IPGs.

Budowle [*8*] described the presence of secondary Gc bands (Gc-actin complex) in Gc subtyping from bloodstains using a mixture of carrier ampholytes pH 4.5 to 5.4 and pH 4 to 6 and the separator *N*,*N*-bis(2-hydroxyethyl)-2-amino-ethanesulphonic acid (BES).

In relation with the detection methods used in the identification of Gc subtypes derived from bloodstains, the most useful technique is the immunofixation with monospecific anti-Gc antisera followed by staining with Coomassie blue or Ponceau S [*9*]. To increase the sensitivity and reduce the cost, alternative procedures have been developed. These include the silver staining of immunofixed Gc on acetate membranes [*5*], and other more sensitive and promising methods, such as detecting the focused Gc bands by enzyme immunoassays after blotting to immobilizing matrices [*10,11*].

This paper presents the application of a rapid and highly sensitive isoelectric focusing method to the identification of Gc phenotypes in bloodstains, by using micro-ultrathin polyacrylamide gel (interelectrode distance: 50 mm) containing a mixture of the separators HEPES and *N*-(2-acetamido)-2-aminoethane sulphonic acid (ACES) with narrow range pharmalytes (pH 4.5 to 5.4) followed by immunoblotting.

Materials and Methods

Equipment and Reagents

Isoelectric focusing was carried out using Pharmacia equipment (flatbed apparatus FBE 3000, constant power supply ECPS 3000/150, and volthour integrator VH-1).

The following reagents were used: acrylamide (Merck), *N*,*N'*-methylenediacrylamide (Merck), sucrose (Merck), ammonium persulphate (Merck), pharmalytes pH: 4.5 to 5.4 and 2.5 to 5 (Pharmacia), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) (Sigma), *N*(2-acetamido)-2-aminoethanesulphonic acid (ACES) (Sigma), urea (Merck), Tween 20 (Merck), gelatin (Difco), 4-chloro-1-naphtol (Merck), bovine serum albumin (BSA) (Behring), anti-human Gc-globulin (Dakopatts), and swine anti-rabbit immunoglobulins/HRP (Dakopatts).

Bloodstains Preparation and Extraction

Bloodstains were prepared on cotton cloth from donors of known Gc types. Thirty donors were used, and all the common Gc phenotypes were examined except Gc 1F (Gc 1S, $n = 7$; Gc 2, $n = 5$; Gc 2-1F, $n = 4$; Gc 2-1S, $n = 6$; and Gc 1S-1F, $n = 8$).

The resulting 30 bloodstains were allowed to dry and kept at room temperature. The samples were typed weekly over a 5-week period. A 20-mm^2 piece of bloodstained cloth was extracted with 30 μL of 6*M* urea for 1 h at room temperature. The extract was separated from the cloth by centrifugation at 1800 rpm for 10 min.

Micro-Ultrathin Polyacrylamide Gel Preparation

The acrylamide stock solution composition was 6.2% T, 3.2% C, and sucrose 12%. The amount of carrier ampholytes and separators added to the stock solution were as follows:

6% (v/v) of pH 4.5 to 5.4 pharmalyte,

0.5% (v/v) of pH 2.5 to 5 pharmalyte,
1.3% (w/v) of HEPES, and
0.6% (w/v) of ACES.

After the addition of separators and carrier ampholytes, the mixture was degassed for 5 min. To initiate polymerization, 10 μL of ammonium persulphate (200 mg/mL) were added per 3 mL of stock solution. The gels were cast onto silanized glass plates (60 by 50 by 1 mm) using the capillary technique [*12*]. The final gel dimensions were 55 by 40 by 0.25 mm, and 1 mL of mixture was sufficient per plate. Polymerization was carried out at 37°C for 15 to 20 min.

Isoelectric Focusing Procedure

Isoelectric focusing was performed using Pharmacia equipment (FBE 3000, ECPS 3000/150, and VH-1).

No electrode solutions were required, as previously described [*13*], and the platinized titanium electrodes rested directly on the gel surface with an interelectrode distance of 50 mm.

The gels were prefocused (1 W, 350 V, 15 mA) for 100 volthours.

Bloodstain extracts (4 μL) were applied on the prefocused gels using pieces of Whatman No. 3 filter paper (4 by 3 mm) at a distance of 0.5 cm from the cathode. Focusing was continued for 15 min with the same settings as prefocusing. After removing sample applicators the gels were focused at 1200 V, 2.5 W, and 15 mA maximum settings until 1600 volthours were reached. The total focusing time to reach the desired volthours (80 to 120 min) depended on the number of plates focused at the same time.

Immunoblotting Procedure

Focused Gc bands were transferred to two different kinds of immobilizing matrices by capillary blotting [*14*]. The immobilizing matrices used were nitrocellulose (NC) membranes (Millipore) and polyvinylidene difluoride (PVDP) membranes (Millipore).

A NC membrane (4 by 3 cm) presoaked in phosphate buffered saline (PBS) pH: 7.2 was laid on top of the gel and covered with a prewetted sheet of filter paper presoaked in PBS and 1 cm of paper towels. The pile was compressed evenly under a 500-g weight for 30 min. Because of its hydrophobic nature, the PVDF membranes were first wetted in a small volume of methanol for 1 to 2 s, then placed in 300 mL of deionized water for 5 min to remove the methanol. Following this step, the PVDF membranes can be used in capillary blotting in the same fashion as described for NC membranes.

After capillary blotting, the membrane was soaked for 1 h in PBS containing 1% gelatin to block the remaining protein binding sites. Then the membrane was incubated for 2 h with 3 mL of rabbit anti-Gc diluted 1:100 in PBS containing 0.02% Tween 20 and 2% bovine serum albumin (BSA). After washing three times for 10 min in PBS containing 0.05% Tween 20, the membrane was incubated overnight with 3 mL of swine anti-rabbit Igs peroxidase conjugated diluted 1:500 in PBS containing 0.02% Tween 20 and 2% BSA. The same washing specified before was repeated. Finally, the peroxidase activity was stained by soaking the membrane in a mixture solution consisting of 30 mg of 4-chloro-1-naphtol in 10 mL of methanol, 50 mL of Tris-HCl buffer pH: 7.4, and 30 μL of 30% hydrogen peroxide. The reaction was stopped with water.

Results and Discussion

Figure 1 shows some of the most common Gc subtypes derived from laboratory prepared bloodstains that were clearly resolved by separator isoelectric focusing in micro-ultrathin

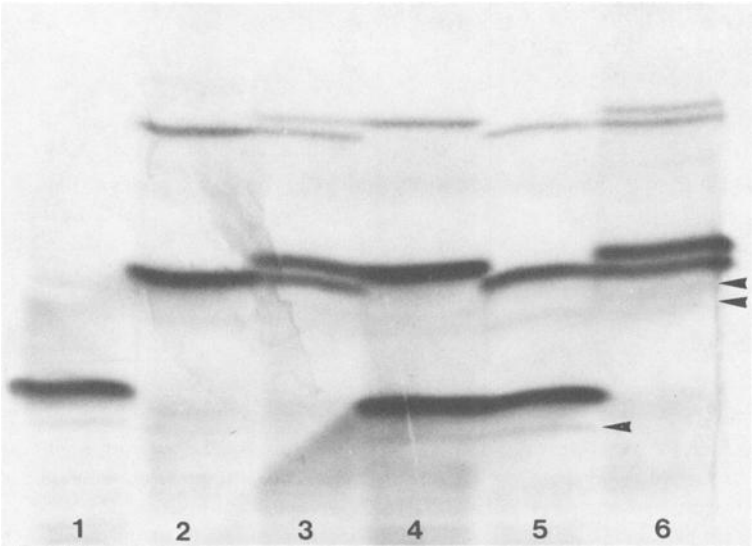

FIG. 1—*Demonstration of Gc phenotypes derived from bloodstains as analyzed by IEF in micro-ultrathin polyacrylamide gel containing pH 4.5 to 5.4 pharmalytes and the separators HEPES and ACES followed by immunoblotting on PVDF membranes. From left to right.* (1) *2,* (2) *1S,* (3) *1F-1S,* (4) *2-1F,* (5) *2-1S, and* (6) *1F-1S. The arrows indicate some secondary Gc bands (anode at the top).*

polyacrylamide gel. The resolution obtained by this method is similar to those reported by Westwood [*5*]. The Gc 1F and Gc 1S bands were separated by approximately 1 mm.

The addition of HEPES and a small amount of ACES provide a slightly better separation between Gc 1F and Gc 1S bands than the separation achieved only with HEPES when using micro-ultrathin polyacrylamide gel with an interelectrode distance of 50 mm.

The secondary Gc band patterns, recently described by Budowle [*8*], were also observed in this study (Fig. 1). These secondary Gc band patterns result from Gc-actin complex even after bloodstain extraction with 6*M* urea.

All 30 bloodstains were correctly subtyped over a 5-week period. Presently, subtyping is being investigated for a longer aging period.

Figure 2 shows the sensitivity limit of the two-step enzyme immunoassay method described in this article. The final dilution was 1 : 320 at which a standard mixture containing Gc 2, Gc 1F, and Gc 1S bands gave a clear pattern to be detected. Assuming that the normal concentration of Gc in human plasma is 300 to 600 μg/mL [*15*], the mean is 450 μg/mL. If the value of 450 μg/mL is taken, this represents a detection limit of approximately 5.6 ng, since only 4 μL of the bloodstain extract is applied to the gel.

In relation with the two kinds of immobilizing matrices used in this study, we have observed that instead of using NC membranes the use of PVDF membranes results in higher sensitivity in Gc subtyping, since PVDF membranes provide superior retention of bound proteins.

On the other hand, we have also observed in preliminary experiments that the use of Tween-20 as a blocking agent, instead of gelatin, reduces dramatically the detection limit of Gc subtypes. This is because Tween-20 rinsed off the proteins from the membrane [*16*].

Conclusions

The combination of separator isoelectric focusing in a narrow pH gradient (pH: 4.5 to 5.4) and the use of miniaturized gels with an interelectrode distance of 50 mm offers a fast and economic method for Gc subtyping in bloodstains.

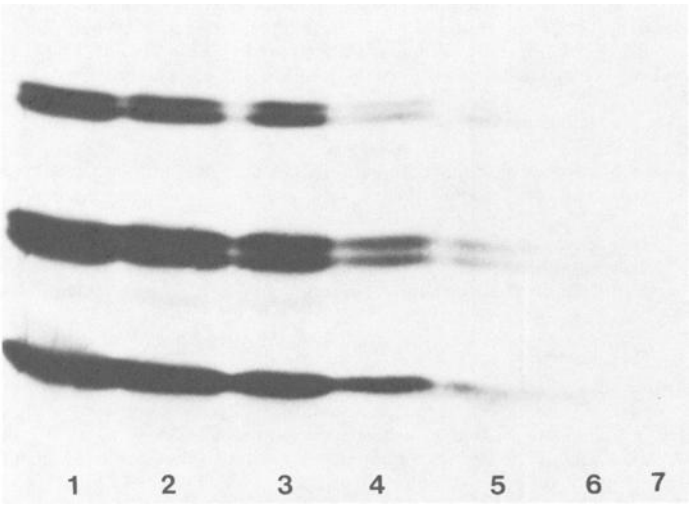

FIG. 2—*Series of dilutions of a standard plasma mixture containing Gc 2, Gc 1F, and G 1S bands separated by IEF and detected by enzyme immunoassay after blotting to PVDF membranes. The dilutions from left to right are:* (1) *1·20,* (2) *1:40,* (3) *1 80,* (4) *1·160,* (5) *1:320,* (6) *1·640, and* (7) *1·1280 (anode at the top).*

The use of enzyme immunoassay after blotting to an immobilizing matrix to detect Gc subtypes is a more sensitive method than immunofixation even after silver staining [5]. The immunoblotting method described is also more economical than immunofixation because very diluted antisera can be used. Furthermore, the immunoblotting technique produced sharper protein bands because the proteins were immobilized on the membrane and no diffusion occurred.

In conclusion, the use of separator isoelectric focusing in micro-ultrathin polyacrylamide gel followed by immunoblotting is suggested as a fast, economical, and highly sensitive method to the identification of Gc subtypes derived from bloodstains.

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