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The Use of Separator Isoelectric Focusing in Micro-Ultrathin Polyacrylamide Gels in the Characterization of Some Polymorphic Proteins of Forensic Science Significance

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ABSTRACT: The identification of phenotypes of erythrocyte acid phosphatase (EAP), esterase D (EsD), group specific component (Gc), and alpha-1-antitrypsin (PI) by separator isoelectric focusing in micro-ultrathin polyacrylamide gels (interelectrode distance: 45 mm) is described. The protein patterns obtained are compared favorably with the patterns seen by isoelectric focusing in conventional polyacrylamide gel dimensions (interelectrode distance: 110 to 120 mm). The technique described allows greater stability of pH gradients and is a fast and economic method.

KEYWORDS: pathology and biology, genetic typing, serology, paternity, protein polymorphism, separator isoelectric focusing, micro-ultrathin polyacrylamide gel, erythrocyte acid phosphatase, esterase D, group specific component, alpha-1-antitrypsin

The isoelectric focusing technique (IEF) utilizing different supporting media was developed during the 1960s [*1-5*]. As a result of its high resolution capacity and reproducible protein patterns, IEF has been widely accepted for the determination of protein polymorphisms in genetic investigations and in forensic serology [*6-10*]. The comparison of this technique with conventional electrophoretic methods has revealed additional information concerning at least 16 isoprotein and 9 isoenzyme markers [*11*].

The advent of isoelectric focusing in a mixture of carrier ampholytes (CA) and Zwitterionic buffers or separators (separator-IEF) [*12*] has provided a simple method for producing narrow pH gradients in the separation of proteins of forensic science interest [*13-20*].

On the other hand, the use of miniaturized slab systems in IEF (micro-IEF) has been described as a fast and highly sensitive technique [*21-26*].

This paper presents the application of separator isoelectric focusing in micro-ultrathin polyacrylamide gels to the identification of phenotypes of erythrocyte acid phosphatase (EAP), esterase D (EsD), group specific component (Gc), and alpha-1-antitrypsin (PI) in human blood.

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Materials and Methods

Equipment and Reagents

Isoelectric focusing was carried out using Pharmacia equipment (flat bed 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); riboflavin-6'-phosphate (LKB, Bromma); pharmalytes pH: 4 to 6.5, pH = 4.2 to 4.9 (Pharmacia); ampholines pH = 5 to 8 and pH 4 to 6 (LKB); *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid (HEPES) (Sigma); *N*-(2-acetamido)-2-aminoethanesulphonic acid (ACES) (Sigma); Coomassie blue R-250 (LKB-, Bromma); 5-sulfosalicylic acid (Merck); trichloroacetic acid (Merck); 4-methylumbelliferyl-acetate (Sigma); 4-methylumbelliferyl-phosphate (Sigma), and DL-dithiothreitol (Sigma).

Samples

Blood samples came from 36 presumptive father-mother-child trios involved in cases of disputed paternity in Spain during the years 1985 through 1986.

EAP and EsD were tested using fresh red blood cells washed three times with isotonic saline solution and lysed by freezing at −40°C. The lysates were diluted 1:1 with dithiothreitol 0.05*M* 30 min before typing.

Gc and PI were tested using serum or EDTA plasma stored at −40°C before analysis and used without previous treatment.

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, depending on the protein investigated, were as follows:

EAP: 4% (v/v) of pH 5 to 8 LKB ampholine,
EsD: 4% (v/v) of pH 4 to 6.5 pharmalyte + 3% (w/v) of HEPES,
Gc: 5% (v/v) of pH 4 to 6 LKB ampholine + 3% (w/v) of HEPES, and
PI: 6% (v/v) of pH 4.2 to 4.9 pharmalyte + 3% (w/v) of ACES.

After addition of separators and carrier ampholytes, the mixture was degassed for 5 min and 2% of a riboflavin solution (200 mg in 100 mL of distilled water) was added. The mixture was poured onto a molding chamber to cast gel layers of 250-μm thickness over silanized glass plates measuring 55 by 50 by 1 mm. The final gel dimensions were 50 by 40 by 0.25 mm and 1 mL of mixture or less was sufficient per plate.

Photopolymerization was carried out under an ultraviolet (UV) lamp for 30 min to 1 h.

Isoelectric Focusing Procedure

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

No electrode solutions were required and the platinized titanium electrodes (1.6 mm in diameter) rested directly on the gel surface with an interelectrode distance of 45 mm.

The gels were prefocused (1.5 W, 350 V, 10 mA) for 100 V/h.

Samples were applied for 15 min on the prefocused gels using pieces of Whatman No. 3 filter paper (4 by 4 mm). The papers were placed at a distance of 0.5 cm from the cathode for ESD, Gc, and PI typing and at a distance of 0.5 cm from the anode for EAP typing.

The focusing conditions for each protein investigated are shown in Table 1, where the time includes the 15 min of sample application.

TABLE 1—*Focusing conditions of EAP, EsD, Gc, and PI in micro-ultrathin polyacrylamide gel (interelectrode distance: 45 mm).*

	Maximum Voltage, V	Maximum Power, W	Maximum Current, mA	Separation Time, min	Total Volthours
EAP	450	1.5	15	35	400-450
EsD	550	1.8	15	45	600-650
Gc	650	2	15	70	750-800
PI	850	2.5	15	100	1200-1300

Enzyme Visualization and Protein Detection

EAP isoenzyme bands were visualized with Whatman 3MM paper soaked in 4-methylumbelliferyl-phosphate at a concentration of 1 mg/mL in 0.05*M* sodium citrate buffer at pH 5.0 and laid on the gel surface. After incubation for 5 to 10 min at 37°C the gel was viewed under UV light (365 mm) (Fig. 1).

EsD isoenzyme bands were visualized by laying on the gel surface a Whatman 3MM paper previously soaked in a 4-methylumbelliferylacetate solution at a concentration of 1 mg/mL in 0.1*M* sodium acetate buffer pH 7.5. After incubation for 5 to 10 min at 37°C the gel was viewed under UV light (365 nm) (Fig. 2).

The Gc band pattern was visualized by simple precipitation in 5% aqueous sulfosalicylic acid solution. Gc appears as intense white band precipitates when sidelighted slabs are observed on a dark background (Fig. 3).

The PI band pattern was visualized after precipitation in 12% aqueous trichloroacetic solution and stained with Coomassie blue R 250 (Fig. 4).

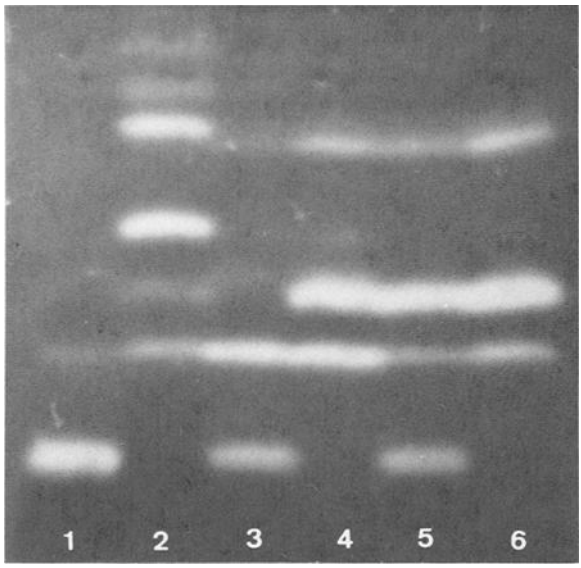

FIG. 1—*Demonstration of EAP phenotypes as analyzed by IEF in micro-ultrathin polyacrylamide gel (pH range 5 to 8). From left to right:* (1) *AA,* (2) *CC,* (3) *CA,* (4) *CB,* (5) *BA, and* (6) *BB. (Anode at the top.)*

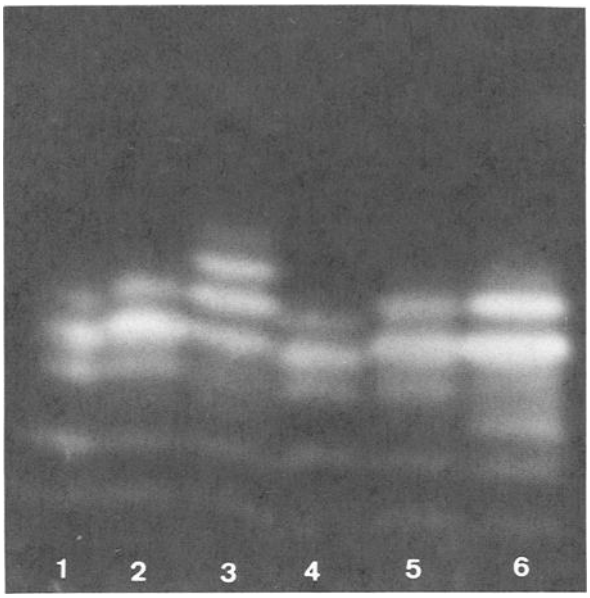

FIG. 2—*Demonstration of EsD phenotypes as analyzed by HEPES-SIEF in micro-ultrathin polyacrylamide gel (pH range 4 to 6.5). From left to right:* (1) *2-2,* (2) *2-1,* (3) *5-1,* (4) *2-2,* (5) *2-1, and* (6) *1-1. (Anode at the top.)*

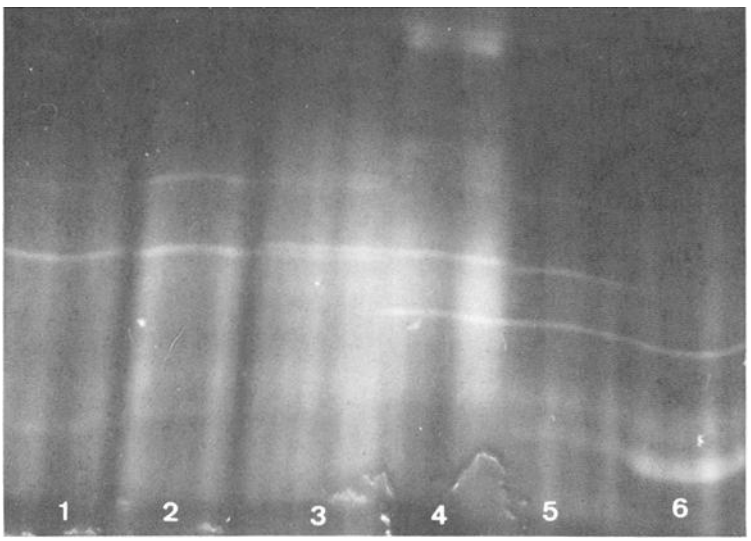

FIG. 3—*Demonstration of Gc phenotypes as analyzed by HEPES-SIEF in micro-ultrathin polyacrylamide gel (pH range 4 to 6). From left to right:* (1) *1F-1F,* (2) *1S-1S,* (3) *1S-1F,* (4) *2-1F,* (5) *2-1S, and* (6) *2-2. (Anode at the top.)*

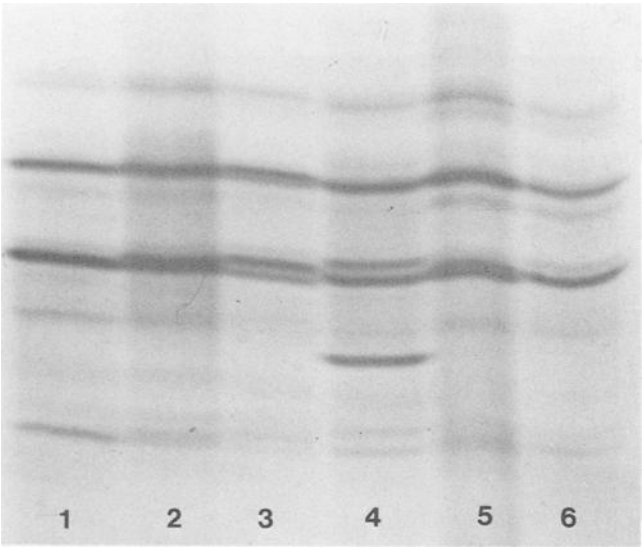

FIG. 4—*Demonstration of some PIM subtypes as analyzed by ACES-SIEF in micro-ultrathin polyacrylamide gel (pH range 4.2 to 4.9). From left to right:* (1) *M2M2,* (2) *M2M3,* (3) *M1M2,* (4) *M3S,* (5) *M1M3, and* (6) *M1M1. (Anode at the top.)*

Results and Discussion

Figure 1 shows the band pattern obtained by IEF in micro-ultrathin gel for the six common EAP phenotypes. The use of ultrathin gels facilitates rapid substrate penetration during isoenzyme visualization and gives a very sharp isoenzyme pattern and a clear distinction of the different phenotypes.

Some of the more common EsD phenotypes (1-1, 2-1, 2-2, and 5-1) are shown in Fig. 2. The combination of HEPES-SIEF with the use of micro-ultrathin gel gives better results in the differentiation of phenotypes, with wider corridors between the isoenzyme bands and a sharper isoenzyme pattern when compared with the standard IEF separation without the use of separators used in our laboratory [*27*].

The pattern of Gc phenotypes obtained by HEPES-SIEF in micro-ultrathin polyacrylamide gel (Fig. 3) also compares favorably with the pattern seen by IEF in conventional polyacrylamide gel dimensions with a clear separation between 1S and 1F bands.

The band patterns of several PIM subtypes obtained after ACES-SIEF in micro-ultrathin polyacrylamide gel are illustrated in Fig. 4.

The results obtained with this method are comparable to those previously published [*28,29*] where ACES-SIEF in conventional polyacrylamide gel dimensions was used. As can be seen in Fig. 4, the subtypes M1M2, M1M3, and M2M3 have a clearly separated double band pattern in the m6 and m8 region.

Conclusions

The most important advantages of the technique described in this article are:

1. The use of platinized electrodes directly on the gel without electrode solutions eliminated a potential source of band disturbances and provided better stability of the pH gradi-

ents. This is particularly important when using narrow pH gradients to ensure that proteins reach their isoelectric endpoints.

2. The use of miniaturized polyacrylamide gels with an interelectrode distance of 45 mm is a fast technique in comparison with the gel dimensions usually cast in standard IEF (interelectrode distance: 110 to 112 mm).

This is not only because polymerization, focusing, and stain/destain times are shorter, but also as a result of the gel dimensions used and the dimensions of the flat bed apparatus used (FBE 3000, Pharmacia) it is possible to run four different gels at the same time. Thus, according to the parameters shown in Table 1, we can test four different protein polymorphisms in 2 h.

On the other hand, the use of miniaturized slab systems substantially reduces the amounts of reactives needed for gel preparation, especially the carrier ampholytes and the reactives used in protein band visualization.

In conclusion, the separator isoelectric focusing in micro-ultrathin polyacrylamide gel is suggested as a fast, economic, and high resolution technique of interest in forensic serology and especially in paternity testing.

The applicability of this procedure in bloodstain analysis is presently under investigation in our laboratory and it will be the subject of further publication.

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