ISOELECTRIC FOCUSING IN MINIATURIZED GELS: APPLICATION TO GC, PI, Tf AND ORM SUBTYPING IN CENTRAL SPAIN

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INTRODUCTION

Since the introduction of isoelectric focusing (IEF) for the analysis of protein polymorphisms of forensic science interest, several technical modifications have been developed to improve its reproducibility and resolution capacity. These include: IEF in a mixture of "separators" and carrier ampholytes (separator-IEF; SIEF) (Caspers et al. 1977; Gill et al. 1984); IEF in immobilized pH gradients (IEF-IPG) (Bjellquist et al. 1982; Cleve et al. 1982) and IEF in IPG-CA supplemented gels (hybrid-IEF; HIEF) (Atland et al. 1985).

On the other hand, the detection of focused bands by enzyme-immunoassay after blotting to immobilizing matrices (immunoblotting), has become a useful tool in forensic science practice due to its high sensitivity and feasibility (Tamaki 1986).

In this study the GC, PI, Tf and ORM-1 genetic variants were investigated by SIEF and/or HIEF using miniaturized gels. The subtypes were detected by immunoblotting in all cases but PI. The distribution of subtypes and the allele frequencies found in Central Spain are shown.

MATERIAL AND METHODS

Plasma samples were obtained from unrelated individuals living in Madrid (n=340 for GC, Tf and PI subtyping and n=315 for ORM subtyping). Samples were stored at - 40o C until used for a maximun period of 6 months. Sample preparation, IEF procedures and detection methods are shown in Table 1.

RESULTS

Figures 1 to 4 show the banding patterns of the GC, PI, Tf and ORM phenotypes respectively as observed by SIEF and/or HIEF.

Tables 2 to 5 show the distribution of GC, PI, Tf and ORM phenotypes respectively observed in Madrid (Central Spain). Allele frequencies are also shown.

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TABLE 1

IEF PROCEDURES (1)

PROTEIN	SAMPLE TREATMENT	SIEF	HIEF	DETECTION METHODS (2)
GC	Plasma samples diluted 1:50 with 6 M urea	2% Pharmalyte 4.5-5.4 1.2 % HEPES, 0.6 % ACES	IPG: 4.8-5.2 Rehydration solution: 2% Pharmalyte 4.5-5.4 0.6 % HEPES,20 % sucrose	- Immunoblotting: * 1st Ab: rabbit anti-human GC (1:500) * 2nd Ab: swine anti-rabbit IgG HRP or AP conjugated (1:1000)
PI	Plasma samples without previous treatment	---	IPGs: 4.35-4.75 4.35-4.55 Rehydration solution: 2 % Pharmalyte 4.2-4.9 0.8 % ACES,20 % sucrose	- Coomasie briliant blue
Tf	Plasma samples diluted 1:7 with 0.15 % ferrous amonium sulfate and incubated 16 h at 4o C	2 % Pharmalyte 4-6.5 1.2 % HEPES	IPG: 5.2-5.7 Rehydration solution: 2 % Pharmalyte 4-6.5 20 % sucrose	- Coomasie briliant blue - Immunoblotting: * 1st Ab: rabbit anti-human Tf (1:1000) * 2nd Ab: swine anti-rabbit IgG HRP
ORM	10 µl of plasma + 10 µl of neuraminidase (C. perfringens, 1 U/mg) at 37o C for 16 h.	1 % Pharmalyte 4.5-5.4 1 % Pharmalyte 4.2-4.9 1 % ACES	IPG: 4.4-5.2 Rehydration solution: 1 % Pharmalyte 4.5-5.4 1 % Pharmalyte 4.2-4.9 0.8 % ACES, 20 % sucrose	- Immunoblotting: * 1st Ab: rabbit anti-human ORM (1:1000) * 2nd Ab: swine anti-rabbit IgG HRP conjugated (1:1000)

(1) The composition of the miniaturized polyacrylamide gels (50-100 x 60 x 0.25-0.5 mm) was 6.2 % T and 3.2 % C. Gels for SIEF and IPG matrices were cast as previously described (Alonso, 1988; Alonso, 1989). SIEF was performed using Pharmacia equipment (FBE 3000, RCPS 3000/150 and VH-1) and HIEF was performed using LKB equipment (2117-003 Multiphor II, 2297 Macrodrive 5 and 2219 Multitemp II). The platinized electrodes rested directly on the gel surface with an interelectrode distance of 55 mm. Gels were prefocused at a low voltage (SIEF: 30-60 V/cm; HIEF: 90-120 V/cm) during 20 min. Samples were applied on the prefocused gels using pieces of Whatmann Nº 3 filter paper (4 x 3 mm) at 0.5 cm from the cathode and IEF was continued for 20 min. with the same settings as prefocusing. Sample applicators were then removed and gels were focused adjusting the power settings to obtain 130-180 V/cm (SIEF) or 300-380 V/cm (HIEF). The total focusing time was 120-140 min. for SIEF and 200-360 min. for HIEF.

(2) Immunoblotting was carried out by simple difussion from the gel to PVDF membranes (Millipore) and focused bands were detected on themembrane by a two-step enzyme-immuno-assay as previously described (Alonso 1988). Antiserums were purchased from Dakopatts (Denmark).

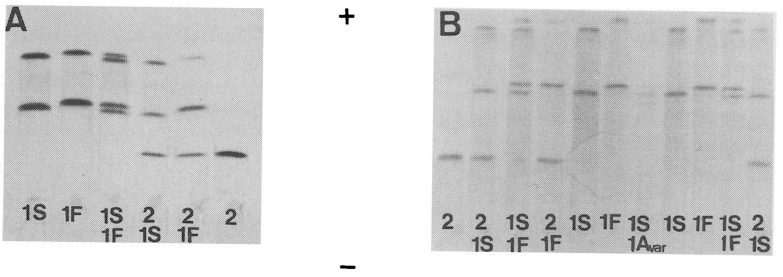

Fig. 1. GC phenotypes analyzed by A) SIEF and B) HIEF in miniaturized polyacrylamide gels.

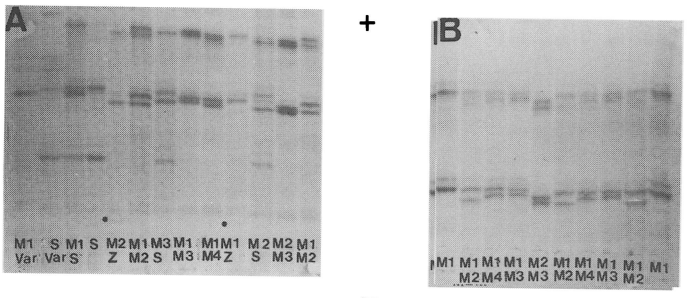

Fig. 2. Pl phenotypes analyzed by HIEF in miniaturized polyacrylamide gels: A) IPG=4.35-4.75 B) IPG=4.35-4.55.
p

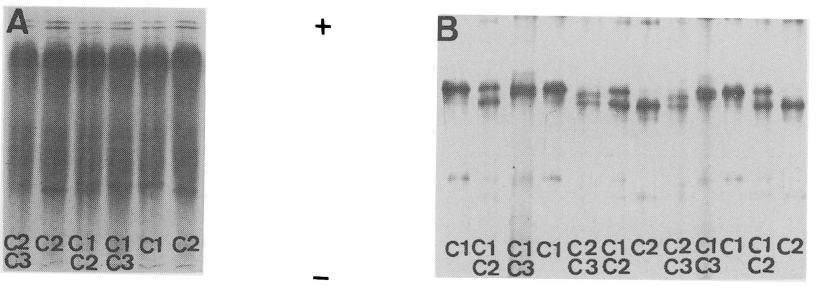

Fig 3. Tf phenotypes analyzed by A) SIEF and B) HIEF in miniaturized polyacrylamide gels.

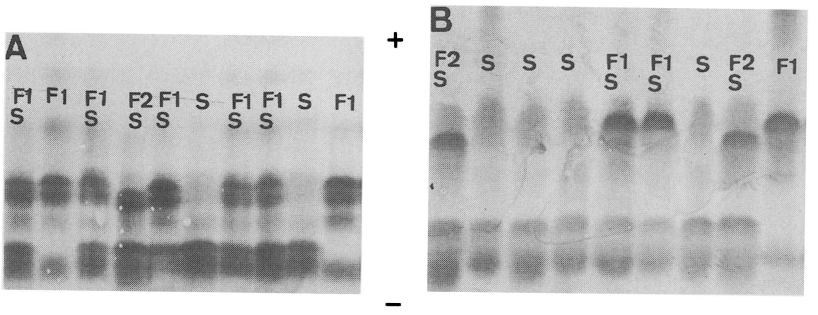

Fig.4. ORM-1 phenotypes analyzed by A) SIEF and B) HIEF in miniaturized polyacrylamide gels.

Table 2. Distribution of GC phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
1S	116	114.14	GC * 1S = 0.5794
2-1S	112	109.49	GC * 2 = 0.2779
1S-1F	48	55.40	GC * 1F = 0.1397
2	25	26.25	GC * $1A_{VAR}$= 0.0029
2-1F	27	26.30	
1F	10	6.63	
$1s-1A_{VAR}$	2	1.14	
TOTAL	340	339.35	

$\Sigma\ I^2 = 3.51367$; df = 3; P > 0.30

Table 3. Distribution of PI phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
M1M1	114	120.61	PI * M1 = 0.5956
M1M2	73	63.14	PI * M2 = 0.1559
M1M3	48	45.28	PI * M3 = 0.1118
M1S	48	45.28	PI * M4 = 0.0132
M2M3	13	11.85	PI * S = 0.1118
M2	6	8.26	PI * Z = 0.0073
M2S	5	11.85	
M1M4	5	5.34	
M3S	7	8.49	
S	7	4.25	
M3	4	4.25	
M2Z	3	0.77	
M4	2	0.059	
M1Z	2	2.95	
$M1VAR_{anod}$	1	1.78	
$SVAR_{anod}$	2	0.3	
Others	0	5.42	
TOTAL	340	340	

$\Sigma\ I^2 = 7.28$; df = 4; P > 0.10. Phenotypes with n(exp) below 5 were combined for I^2 calculation.

Table 4. Distribution of Tf phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
C1	218	213.59	Tf * C1 = 0.7926
C1C2	78	86.39	Tf * C2 = 0.1603
C1C3	24	23.77	Tf * C3 = 0.0441
C2	13	8.74	Tf * C6 = 0.0029
C2C3	4	4.81	
C3	1	0.66	
C1C6	1	1.56	
C2C6	1	0.32	
C3C6	0	0.086	
C6	0	0.0028	
TOTAL	340	339.93	

$\Sigma\ I^2 = 3.0118$; df = 2; P > 0.2. Phenotypes with n(exp) below 5 were combined for I^2 calculation.

Table 5. Distribution of ORM-1 phenotypes and allele frequencies in Central Spain.

Phenotype	Observed	Expected	Allele frequencies
F1S	157	146.46	ORM-1 * F1 =0.6206
F1	117	121.32	ORM-1 * F2 =0.0047
S	38	44.20	ORM-1 * S =0.3746
F2S	3	1.109	
F1F2	0	1.83	
F2	0	0.006	
TOTAL	315	314.92	

$\Sigma\ I^2 = 1.7829$; df = 1; P > 0.10. Phenotypes with n(exp) below 5 were combined for I^2 calculation.

CONCLUSIONS

- SIEF using miniaturized polyacrylamide gels as well as HIEF in small IPG matrices (focusing distance: 50-55 mm) have been shown as reliable and reproducible isofocusing methods with a resolution capacity similar to that achieved by IEF in large gels (focusing distance: 100-120 mm).

- The use of small gels is a fast and economic method that allows the simultaneous analysis of several polymorphic proteins because it is possible to run up to 4 different gels (45 x 60 x 0.25 - 0.5 mm) at the same time using only one flat bed apparatus.

- Immunoblotting, which has been shown as a specific and sensitive method, is recomended for routinary subtyping of GC, ORM and Tf.

- The distribution of phenotypes found in Madrid for the proteins analyzed in this study are in good agreement with the H-W equilibrium.

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