The Use of Hybrid Isoelectric Focusing for the Detection of Polymorphic Proteins and Enzymes in Bloodstains Analysis

Muñoz-Barús JI, Carracedo A,. López-Rodríguez I, Montiel MD

Dept. Medicina Legal. Facultad de Medicina
Universidad de Santiago de Compostela. Spain

INTRODUCTION

Immobilized pH gradients (IPG) show a number of advantages over IEF with carrier ampholytes (CA). Its power of resolution is 10 times greater, any pH gradient can be created, sample of up to 10 times grater can be loaded and cathodic drift is eliminated (Righetti et al. 1988).
Nevertheless, previous experience with immobilized pH gradients gels has shown them to be insensitive compared to Ampholine gels (Altland et al. 1986). This is a great inconvenience for the detection of polymorphism in minute bloodstains.
Incorporating CA into IPG gels (HIEF) produces improvement of sensitivity because the CA improves the conductivity and prevents the precipitation of protein before separation can be achieved (Altland et al. 1987).
In this paper we report the advantages and disadvantages of HIEF for the analysis of certain proteins (Pi, Tf, ORM) and enzymes (PGM, ACP, ESD) in bloodstains.

MATERIAL AND METHODS

Samples: 30 µL bloodstains (2 weeks old) on cotton cloth.
Extraction: 30 µL 0,05 M DTT.
Pretreatment: Neuraminidase (1U/mL). 12 h. 4 °C for ORM
0.5 M ferrous ammonium sulphate. 12 h. 4 °C for Tf
Dilutions: Doubling dilutions of the bloodstain extracts we prepared to a final dilution of 1:64

HIEF: IPG gels were cast according to Bjellqvist et al. (4) on a GelBond PAG films.
Gel dimensions: 260 x 100 x 0.5 mm.

Advances in Forensic Haemogenetics 3
Edited by H. F. Polesky and W. R. Mayr

Formation of Immobilized pH gradients

Composition of the acidic and basic components of the gradient mixer for the separation of the ACP, ESD, PGM1, Pi, Tf and ORM phenotypes (see * for specific immobilines volumes)

Basic light	mL	Acidic dense	mL
Acrylamide (29.1%) + bis-acrylamide (0.9%)	1.25	Acrylamide + bis-acrylamide	1.25
Glycerol 87%	0.00	Glycerol	2.10
H2O	X	H2O	Y
Total volume	7.50	Total volume	7.50
TEMED (10 mL%)	0.01	TEMED	0.01
Ammonium persulfate (10 g%)	0.01	Ammonium perfulfate	0.01

* Volume (µl) 0.2 M Immobiline pK

Proteins pK	Acidic dense solution			Basic light solution		
	Pi	Tf	ORM	Pi	Tf	ORM
3.6	-	527.5	-	-	142	-
4.6	377	-	354	374	-	433
6.2	-	582.5	-	-	347	-
9.3	121.5	-	131.5	207	-	350

pH range: Pi 4.2-4.6, Tf 5.3-6.3, ORM 4.3-5.1

* Volume (µL) 0.2 M Immobiline pK

Enzymes pK	Acidic dense solution			Basic light solution		
	ACP	ESD	PGM1	ACP	ESD	PGM1
3.6	351	176	338	87.5	-	91
4.6	127	375	-	61.5	431.5	-
6.2	208	431.5	408.6	65.5	431.5	353.5
7.0	66.5	-	-	172.5	-	-
8.5	173	-	-	173	-	-
9.3	-	-	-	-	101	-

pH range: ACP 5-8, ESD 4.8-5.8, PGM1 5.6-6.6

HIEF gel casting

Polymerization: 15 min at room temperature + 1 h at 50 °C
Washing: 6 x 10 min with distilled water. Drying
Rehydration (mold method) more than 1 h

	ACP	ESD	PGM	Pi	Tf	ORM
Ampholine	4-6/6-8	4-6	5-7	3.5-5	5-7	4-6
%	1%	2%	1%	0.5%	0.5%	1%

Conditions

Electrode solutions	+ 10 mM glutamic acid
	- 10 mM NaOH
Sample application	+ PGM, ACP
	- ESD, Pi, ORM, Tf

Focusing conditions: 7 W, 4 mA, 3500 V for 3 h
Staining: appropriate substrates for enzymes and silver staining for proteins

RESULTS AND DISCUSSION

Pi, Tf C, ORM and PGM phenotypes are much better distinguishable by HIEF than by IEF with CA since pH range can be considerably reduced .
Bands are sharper and straighter using HIEF particularly with contaminated bloodstains.
HIEF gave good results to the final extract solution of 1:64 for Pi and 1:32 for Tf, ORM, PGM, ACP and ESD .
For ACP and ESD, HIEF has serious handicaps: ACP typing by HIEF is expensive (five different Immobilines must be used), the ACP*A allele is not perfectly separated from Hb, and although the ESD*5 allele can be better separate with HIEF, the alleles ESD*1 and ESD*2 are not well distinguished .
HIEF is clearly the method of choice for the detection of Pi, Tf, ORM and PGM1 in bloodstains.
The resolution power of HIEF (and IPGs) is illustrated with the demonstration of a new PGM variant, which appeared to be slightly cathodal to PGM1 a8 and anodal to PGM1 a1, assuming as lineal the pH range of Dykes and Polesky (1981), but without repetitions in the cathodic side. Family studies confirms the codominant mode of inheritance of this variant.

REFERENCES

Altland K, Eckaerdstein A, Banzhoff A, Wagner M, Rossmann U, Hackler R, Becher P (1987) Hybrid isoelectric focusing: adsorption of proteins onto immobilized pH gradient matrices and desorption by carrier ampholytes. Electrophoresis 8:52-62

Altland K, Hackler R, Rossmann U (1986) Avoiding liquid exudation on the surface of rehydrated gels used for hybrid isoelectric focusing in carrier ampholyte supplemented immobilized pH gradients. Electrophoresis 7:251-259

Bjellqvist B, Ek K, Righetti PG, Gianazza E, Görg A, Westermeier R, Postel W (1982) Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. J.Biochem.Biophis. Methods 6:317-339

Dykes DD and Polesky HF (1981) Comparison of rare PGM1 variants by isoelectric focusing and conventional electrophoresis: Identification of five new variants. Electrophoresis 2:323-326

Righetti PG, Chiari M, Gelfi C (1988) Immobilized pH gradients: effect of salts, added carrier ampholytes and voltage gradients on protein patterns. Electrophoresis 9:65-73