PI, C2, GC, ATIII, PLG Typing in Bloodstains by Hybrid Isoelectric Focusing (HIEF)

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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 greater 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 seminal and 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).

Protein markers *alpha 1-antitrypsine (PI), plasminogen (PLG), Group Specific Component (GC), antithrombine III* (ATIII) and *complement component C2* (C2) are analyzed in series of bloodstains to determinate the usefulness of the hybrid isoelectric focusing (HIEF) for typing electrophoretic markers for forensic material.

MATERIAL AND METHODS

Samples: 30 µL bloodstains (2 weeks old) on cotton cloth.
Bloodstain extraction: 30 µL 0,05 M DTT and doubling dilutions were prepared.
Samples treatment: 10 µL bloodstain extracts were treated with neuraminidase (1 units/mL) for at least 12 h at 4 °C for PLG and ATIII typing.
HIEF: *IPG* gels were cast according to Bjellqvist et al. (1982) on a GelBond PAG films with a gel dimensions of 260 x 100 x 0.5 mm.

FORMATION OF IMMOBILIZED pH GRADIENTS

Composition of the acidic and basic components of the gradient mixer (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	7.5	Total volume	7.50
TEMED (10 mL%)	0.01	TEMED	0.01
Ammonium persulfate (10 g%)	0.01	Ammonium perfulfate	0.01

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* VOLUME (µl) 0.2 M *IMMOBILINE* pK

pK	Acidic dense solut				Basic light solution			
	3.6	4.6	6.2	9.3	3.6	4.6	6.2	9.3
pH 4.1-5.1		356.5		88.5		401.5		329.5
pH 4.2-4.8		377		121.5		373		292
pH 4.5-5.4		367.6		174.3		566.5		510.5
pH 5.7-6.7	299		377.5		80.5		372.5	
pH 5.4-6.4	449.5		508.5		121		314	
pH 5.5-6.5	387.5		451.5		104.5		384	

HIEF GEL CASTING

Polymerization: 15 min room temperature + 1h 50 °C

Washing: 6 x 10 min with distilled water + 1 x 30 min with 2% Glycerol

Drying

Rehydration (mold method) more than 1 h
IPG 4.1-5.1 :2% Ampholine pH 4-6
IPG 4.2-4.8: 2% Ampholine pH 4-6
IPG 4.5-5.4: 1.5% Pharmalyte pH 4.5-5.4
IPG 5.7-6.7: 2% Ampholine pH 5-7
IPG 5.4-6.4: 1% Ampholine pH 5-7
IPG 5.5-6.5: 1% Ampholine pH 5-7

RUNNING CONDITIONS AND STAINING METHODS

Electrode solutions: + 10 mM glutamic acid. - 10 mM N_aOH

Sample application: cathode (PI, GC, ATIII, C2), anode (PLG)

Focusing conditions:
HIEF 4.2-4.8: 7 W, 4 mA, 3500 V for 5 h
HIEF 4.5-5.4: 7 W, 4 mA, 3500 V for 3 h
HIEF 4.1-5.1; 5.7-6.7; 5.5-6.5 and 5.4-6.4: 7 W, 4 mA, 3000 V for 3 h

Coomassie Brilliant Blue R-250 for PI, fixation with Sulfosalicylic acid and stained with Coomassie Brilliant Blue R-250 and immunoprinting with cellulose acetate strips soaked with monospecific *GC*-antiserum and stained with Coomassie Brilliant Blue R-250 [5] for GC, immunofixation with specific antisera and stained with CBB R-250 or silver staining methods for PLG, ATIII and C2.

RESULTS AND DISCUSSION

PIM subtypes obtained with *HIEF* are much more distinguishable by IPGs than by IEF with CA, since the pH range can be considerably reduced. Furthermore, bands are sharper and straighter using *IPGs*, particularly with contaminated bloodstains. Other additional advantages are that *IPGs* are not sensitive to salts, and much more sample can be loaded in *IPGs*. *IPGs* gave good results to the final extract solution of) 1:64 when 10 µL were loaded on the sample paper (equivalent to 0.15 µL of

liquid blood). The sensitivity is similar to IEF with conventional CA and superior to IPG gels without rehydration with CA. This may be due to the solubilizing properties of CA. Although the pH range 4.2-4.8 improved the resolution of PI M subtypes, some PI variants can be lost, and so we usually use, for routine case work, a pH range 4.1-5.1, which partially covers all variants, allowing a clear identification of subtypes Pi M1, M2, M3, and M5.

GC subtypes 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 1:32.

PLG phenotypes are observed by *HIEF* only after immunofixation and CBB or silver staining methods, bands are sharper compared with IEF with CA, and *HIEF* improved the resolution of PLG patterns.

The use of immunofixation and silver staining methods following *HIEF* means that ATIII phenotypes can be detected in 5 µL of two week-old bloodstains extracted with distilled water without concentration procedures.

For C2, only 3 day-old bloodstains can be detected due to activation but phenotypes are clearly distinguished in more recent bloodstains and *HIEF* gave good results to the final extract solution of 1:8 when 10 µL were loaded on the paper.

In conclusion we recommend the routine use of *HIEF* for the detection of these proteins and enzymes on forensic casework because it is clearly the method of choice for the detection of these polymorphic proteins in bloodstains since phenotypes are better distinguished, much more sample can be loaded, bands are straighter and sharper (even with contaminants) and the sensitivity is similar to IEF with CA.

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