Bruce Budowle,[1] *Ph.D. and Anne M. Gambel,*[1] *B.S.*

An Alternative, Effective Substrate for Erythrocyte Acid Phosphatase Phenotype Determinations

REFERENCE: Budowle, B. and Gambel, A. M., **"An Alternative, Effective Substrate for Erythrocyte Acid Phosphatase Phenotype Determinations,"** *Journal of Forensic Sciences*, JFSCA, Vol. 33, No. 4, July 1988, pp. 915-920.

ABSTRACT: A method is described for obtaining nondiffusing, nonfading fluorogenic zymograms for erythrocyte acid phosphatase variants separated by isoelectric focusing. The synthetic substrate 4-trifluoromethylcoumarin phosphate was impregnated into cellulose diacetate membranes and air-dried overnight. After isoelectric focusing, the substrate overlay membrane was rehydrated in 0.05*M* citrate buffer, pH 4.0, lightly blotted, and overlayed on the gel. A 5- to 10-min incubation at 37°C produced intensely fluorescing, light-blue bands on the diacetate membrane. Interaction of the trifluoromethyl group on the substrate with hydrophobic regions of the diacetate membrane impeded diffusion, yielding a permanent zymogram. There were no discrepancies in phenotype determinations using this method when compared with the 4-methylumbelliferyl phosphate assay approach. Further, an increased number of conclusive calls were obtained (91.8 versus 79.5% and 54.1 versus 34.9%) with this new assay when compared with the 4-methylumbelliferyl phosphate substrate on known liquid bloods and questioned dried bloodstains, respectively.

KEYWORDS: forensic science, phosphatases, fluorogenic substrates, isoelectric focusing, cellulose diacetate membranes, erythrocyte acid phosphatase, forensic serology, bloodstains

Erythrocyte acid phosphatase (EAP, E.C. 3.1.3.2) is a genetically controlled polymorphism governed by three autosomal codominant alleles designated A, B, and C [*1,2*]. Historically, continuous zone electrophoresis (CZE) has been the most commonly used approach for separating the EAP variants [*1-7*]. Following electrophoresis, the EAP phenotypes are detected by enzymatic hydrolysis of the fluorogenic substrate 4-methylumbelliferyl phosphate (MUP) [*8*]. A major difficulty inherent in EAP phenotype interpretations lies in the determination of the C, CB, and B phenotypes that rely on subjective comparisons of band intensities [*2,9,10*]. Unfortunately, the EAP bands diffuse at different rates during CZE, making phenotype interpretations difficult. The concentrating effect of isoelectric focusing (IEF) minimizes diffusion and thus overcomes the EAP phenotype interpretation problem observed with CZE. There are several reports in the literature of IEF techniques for separating EAP variants that yield very good results [*11-15*]. Although IEF controls band sharpness, some of the concentrating effect can be lost due to excessive diffusion of the fluorogenic

This is Publication No. 87-6 of the Laboratory Division of the Federal Bureau of Investigation. Names of commercial manufacturers are provided for identification only, and inclusion does not imply endorsement by the Federal Bureau of Investigation. Received for publication 27 July 1987; revised manuscript received 2 Nov. 1987; accepted for publication 13 Nov. 1987.

[1]Research chemist and scientist, respectively, Forensic Science Research and Training Center, Laboratory Division, FBI Academy, Quantico, VA.

substrate during the subsequent visualization step. This can reduce the sensitivity of detection of an EAP-IEF system and over a short period of time (30 min to 1 h) make it difficult, if not impossible, to interpret EAP zymograms. This report describes an alternative assay method for EAP using the synthetic substrate 4-trifluoromethylcoumarin phosphate (HFCP) impregnated into cellulose diacetate membranes (DAMs). This assay minimizes the diffusion effects on the fluorogenic bands, is more sensitive than the MUP assay, and results in a permanent record of the EAP zymogram.

Materials and Methods

Blood specimens were obtained by finger prick from 15 donors of known EAP phenotype. All 6 of the common EAP phenotypes were represented. In addition to these known donor specimens, 122 known liquid blood specimens and 109 questioned bloodstains obtained from cases submitted to the Federal Bureau of Investigation (FBI) Laboratory were analyzed. Bloodstains were prepared as previously described [*16*]. Cuttings (2 by 3 mm) of the bloodstains were extracted in 20 μL of 50m*M* dithiothreitol for 30 min at room temperature with constant agitation. The extracts were absorbed onto 5- by 5-mm applicator tabs (LKB), lightly blotted, and applied 0.4 cm from the anode.

Polyacrylamide gels (5% T, 3% C, 64 by 90 by 0.2 mm) containing pH 5 to 7 Ampholines (LKB) (final concentration of 4% w/v) were cast onto silanized glass plates using the capillary technique on a Bio-Rad ultrathin layer casting tray or by the flap technique [*17,18*]. Ultrathin-layer polyacrylamide gel isoelectric focusing (ULPAGIF) was performed as previously described [*14*].

The substrate overlay membrane was prepared essentially as described by Smith [*19*]. Seven milligrams of HFCP (Enzymes Systems Products, Livermore, CA) were dissolved in one millilitre of deionized water and stored at −20°C. One hundred and twenty-five microlitres of the stock solution were diluted with deionized water to a final volume of 10 millilitres. A DAM (0.2-μm pore, 125 by 125 mm) (supplied by Enzyme Systems Products) was quickly immersed in the substrate solution to ensure even moistening. The DAM was removed from the substrate solution, flipped over, and again placed in the substrate solution. The DAM was air-dried overnight by suspending the membrane from a paper clip mounted on a wire strung across a fume hood. The dry, treated membrane was stored at room temperature between sheets of nonabsorbent onion paper until used. Stability of membranes thus prepared has been reported to be approximately four to five months [*19*].

After ULPAGIF, dry substrate overlay membranes were cut to appropriate dimensions, wetted in 0.05*M* citrate buffer, pH 4.0, lightly blotted, and carefully overlayed on the gel. Care was taken to avoid trapping air bubbles between the membrane and the gel. A glass plate was placed on top, and the entire complex was put in an incubator at 37°C for 5 to 10 min. The glass plate was removed, and the EAP patterns were observed under long-wave ultraviolet (UV) light before and after removing the membrane from the gel. Gels also were assayed using the standard technique of cellulose acetate membranes impregnated with MUP (CAM-MUP) [*12,14*] for comparative purposes. All membranes were removed from the gels within 20 min, air-dried, and maintained at room temperature in protective plastic bags for future evaluations.

Results and Discussion

The EAP phenotypes from the known donor specimens were consistent with both enzyme detection methods. Figure 1 shows that the allelic products of EAP separated by ULPAGIF can be assayed using DAMs-HFCP. The fluorogenic bands were light blue, sharp, and intense. The B allelic product appeared to be slightly more active on the HFCP substrate compared with MUP. Therefore, for interpretational purposes, any patterns produced using

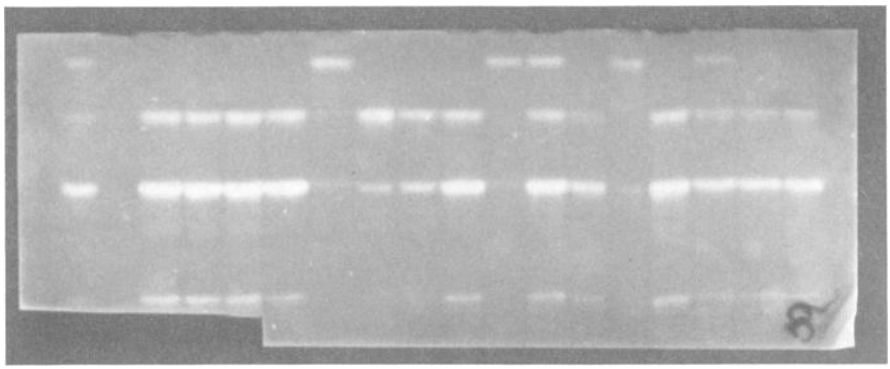

FIG. 1—*Zymogram of EAP developed using HFCP substrate. Phenotypes from left to right are BA, negative, B, B, B, B, A, C, CB, B, A, BA, B, A, B, BA, B, and B. Cathode is at top.*

DAMs-HFCP in which the C and B bands are of equal intensity or when the B band is slightly more intense than the C band should be considered a CB phenotype (Fig. 1). If the B band is less intense than the C band, the phenotype should be considered a C. Further, for the proper evaluation of phenotypes of unknown specimens, CB and A phenotype controls should be included on every gel. The CB control provides a standard for the comparison of relative band intensities, and the A control will ensure that the A band did not migrate off the gel. Figure 2 compares the EAP zymograms from CAM-MUP with DAM-HFCP. Both zymograms were rewetted with water after drying to facilitate observation of the fluorogenic bands. Initially, when the membranes were still on the gels, the bands were intense for both

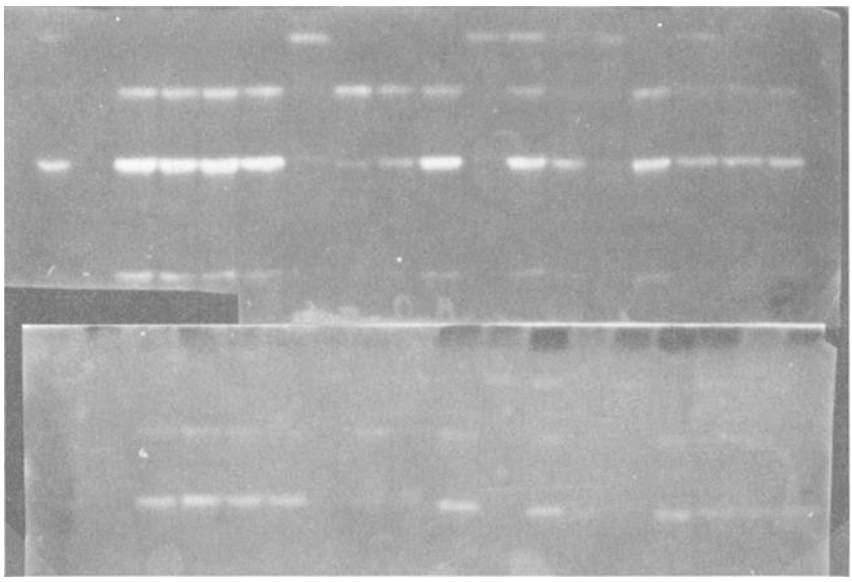

FIG. 2—*Zymogram in Fig. 1* (top) *compared with EAP zymogram developed using MUP substrate* (bottom). *The same specimens were assayed by both assay methods. These zymograms were photographed three months after electrophoretic assay and have been continuously stored at room temperature.*

assays. While, in our experience, dried EAP zymograms resulting from the CAM-MUP approach can be maintained for long periods of time (six months to date), the patterns tend to fade, even with subsequent rewetting. During this study, fluorogenic band intensity on CAM-MUP diminished from one half to one third the original intensity within one week of storage at room temperature. In contrast, the intensity of the bands from the DAM-HFCP did not fade. The patterns were four to five times more intense (visually estimated) than the patterns on CAM-MUP. Further, each subsequent wetting of the CAM-MUP zymogram to facilitate band detection resulted in increased diffusion of the fluorogenic bands, whereas this is minimized with DAM-HFCP. Eventually, this diffusion effect made it difficult to interpret the EAP phenotypes in CAMs-MUP. Additionally, unlike the DAM-HFCP zymograms, when the EAP zymogram developed by the CAM-MUP approach was maintained in a hydrated state on the gel for more than 30 min, phenotyping was no longer possible. The impedence of diffusion of the fluorogenic bands on DAMs-HFCP is most likely due to strong hydrophobic interactions with regions in the DAM and the trifluoromethyl group of the substrate [*19,20*].

By using doubling dilutions of bloodstain extracts, the sensitivity of the DAM-HFCP assay for detecting EAP was determined to be twice that of the CAM-MUP approach. This observation was expected since diffusion is minimized with the DAM-HFCP assay. Since half the quantity of specimen would be required for analysis, more specimen would be available for additional assays. Increased sensitivity of detection can impact upon forensic serology where specimen quantity is often limiting. It should be noted that fuming EAP zymograms on both systems with ammonia can enhance slightly the band intensity. In the DAM-HFCP assay, the band color will shift from blue to green in the presence of ammonia, providing greater contrast between the fluorogenic bands and the background.

Because the FBI Laboratory is interested in the forensic science application of this new assay method, a comparison of the two different assay approaches was made on the 15 known specimens and on the known liquid bloods and questioned dried bloodstains submitted to the FBI Laboratory. To ensure the same amount of specimen was evaluated by both assays, we used equal volumes from the same aliquot of bloodstain extract. There was no discrepancy in phenotype determinations between the DAM-HFCP and CAM-MUP assays. Table 1 displays the comparative efficiency of both assay methods on the case specimens submitted to the FBI Laboratory. For the known liquid blood specimens, conclusive phenotype determinations on 91.8% of the specimens were obtained with the DAM-HFCP assay, compared with 79.5% for the CAM-MUP approach. Conclusive determinations on questioned bloodstains of 54.1 versus 34.9%, respectively, were observed. All specimens, except one, that yielded conclusive results by assaying with CAM-MUP also were conclusive by the DAM-HFCP method. The one specimen was too weak to be conclusively determined by the DAM-HFCP assay. At this time there is no explanation for the observation regarding this one specimen. However, these data suggest that the DAM-HFCP assay is more effective for bloodstain analysis. Note that the specimens from the adjudicated cases were maintained at −20°C for 11 months before this study. The efficacy of both assay methods would be expected to be greater on fresher bloodstains.

TABLE 1—*Comparison of typing results using the HFCP and MUP substrates on forensic science specimens.*

		Conclusive		Inconclusive		No Activity	
Specimens	No.	HFCP	MUP	HFCP	MUP	HFCP	MUP
Known liquid bloods	122	112	97	8	23	2	2
Dried questioned bloodstains	109	59	38	28	45	22	26

During the course of this study, some interesting differences were observed in the interpretation of EAP phenotypes when separated on capillary-poured gels as opposed to flap-poured gels. The original validation of the ULPAGIF of EAP procedure used in this study was performed in gels cast by the flap technique. There appeared to be either an isomeric shift of the C allelic product to that of a B [*21*] or preferential loss of the enzymatic activity of C when EAP was separated in capillary-poured gels. This effect occurred regardless of the fluorogenic substrate used to produce the zymogram. Thus, some C and CB phenotypes can appear as CBs and Bs, respectively, when separated in capillary-poured gels. To evaluate properly EAP phenotypes by ULPAGIF, the use of polyacrylamide gels cast by the flap technique is recommended. Differences in results obtained with the two gel types have been reported. These include inhibition of phosphoglucomutase-1 activity [*22*], effects on silver stain sensitivity [*23*], and in this study, joule heat production (a final wattage of 15 versus 30 W was observed for flap and capillary gels, respectively). These phenomena may be due to polymerization by-product differences between the gels [*24*].

In conclusion, the synthetic substrate HFCP provides a specific and sensitive assay for EAP. Further, when the substrate is impregnated into DAMs, EAP zymograms with intensely fluorescing, light blue bands are produced that do not fade or diffuse. To date, zymograms have been maintained at room temperature for six months with no observable changes in the patterns. The cost of the assay is slightly less than the CAM-MUP method, and the materials are commercially available (Enzyme Systems Products). In addition to offering the convenience of unaffected interpretation over time, a permanent zymogram obviates the need for photography to maintain data. High-resolution photography cannot produce a copy of comparable quality to the actual zymogram [*25*]. Moreover, reducing the need for photography will result in a substantial time and cost savings. Presently, we are investigating a similar approach for assaying esterase D.

References

[*1*] Hopkinson, D. A., Spencer, W., and Harris, H., "Red Cell Acid Phosphatase Variants: A New Human Polymorphism." *Nature*, Vol. 199, 1963, pp. 969-971.

[*2*] Hopkinson, D. A., Spencer, W., and Harris. H., "Genetical Studies on Human Red Cell Acid Phosphatases," *American Journal of Human Genetics*, Vol. 16, 1964, pp. 141-154.

[*3*] Wraxall, B. G. and Culliford, B. J., "A Thin-layer Starch Gel Method for Enzyme Typing of Bloodstains," *Journal of the Forensic Science Society*, Vol. 8, 1968, pp. 81-82.

[*4*] Hoppe, H., Hennig, W., and Brinkmann, B., "Horizontal Polyacrylamide Electrophoresis for the Determination of Serum Protein (Haptoglobin) and Red Cell Enzyme Polymorphism," *Humangenetik*, Vol. 14, 1972, pp. 224-231.

[*5*] Saha, N. and Kirk, R., "An Economical Method for Red Cell Acid Phosphatase Typing Using Polyacrylamide Gel Electrophoresis," *Indian Journal of Medical Research*, Vol. 62, 1974, pp. 174-177.

[*6*] Sorenson, S., "Agarose Gel Electrophoresis of Human Red Cell Acid Phosphatase Isozymes," *Vox Sanguinis*. Vol. 27, 1974, pp. 556-563.

[*7*] Grunbaum, B. and Zajac, P., "Phenotyping of Erythrocyte Acid Phosphatase in Fresh Blood and in Bloodstains on Cellulose Acetate," *Journal of Forensic Sciences*. Vol. 23, 1978, pp. 84-88.

[*8*] Harris, H. and Hopkinson, D. A., *Handbook of Enzyme Electrophoresis in Human Genetics*, Section 3.1.3.2, Elsevier Press, New York, 1976.

[*9*] Spencer, N., Hopkinson. D. A., and Harris, H., "Quantitative Differences and Gene Dosage in Human Red Cell Acid Phosphatase Polymorphism," *Nature*, Vol. 201. 1964, pp. 299-300.

[*10*] Eze, L. C., Tweedie, M. C., Bullen, M. F., Wren, P. S.. and Evans, D. A., "Quantitative Genetics of Human Red Cell Acid Phosphatase," *Annals of Human Genetics*. Vol. 37, 1974, pp. 333-340.

[*11*] Burdett, P. E. and Whitehead, P. H., "The Separation of Phenotypes of Phosphoglucomutase, Erythrocyte Acid Phosphatase, and Some Hemoglobin Variants by Isoelectric Focusing," *Analytical Biochemistry*, Vol. 77, 1977, pp. 419-428.

[*12*] Randall, T., Harland, W. A.. and Thorpe, J. W., "A Method of Phenotyping Erythrocyte Acid Phosphatase by Iso-Electric Focusing," *Medicine, Science and the Law*, Vol. 20, 1980, pp. 43-47.

[*13*] Divall, G. B., "Studies on the Use of Isoelectric Focusing as a Method of Phenotyping Erythrocyte Acid Phosphatase," *Forensic Science International*. Vol. 18, 1981, pp. 67-78.

[14] Budowle, B., "Rapid Electrofocusing of Erythrocyte Acid Phosphatase," *Electrophoresis*, Vol. 5, 1984, pp. 254–255.
[15] Frank, W. E. and Stolorow, M. D., "The Use of Ultrathin Layer Agarose Gels for Phenotyping Erythrocyte Acid Phosphatase by Isoelectric Focusing," *Journal of Forensic Sciences*, Vol. 31, 1986, pp. 1089–1094.
[16] Budowle, B., "Phosphoglucomutase-1 Subtyping of Human Bloodstains on Ultrathin-layer Polyacrylamide Gels," *Electrophoresis*, Vol. 5, 1984, pp. 165–167.
[17] Allen, R. C., "Rapid Isoelectric Focusing and Detection of Nanogram Amounts of Proteins from Body Tissues and Fluids," *Electrophoresis*, Vol. 1, 1980, pp. 32–37.
[18] Radola, B. J., "Ultrathin-layer Isoelectric Focusing in 50–100 μm Polyacrylamide Gels on Silanized Glass Plates or Polyester Film," *Electrophoresis*, Vol. 1, 1980, pp. 43–56.
[19] Smith, R. E., "Identification of Protease Isozymes after Analytical Isoelectric Focusing using Fluorogenic Substrates Impregnated into Cellulose Membranes," *Journal of Histochemistry and Cytochemistry*, Vol. 32, 1984, pp. 1265–1274.
[20] Smith, R. E., "A New Fluorescent Detection System for Identifying Variant Hemoglobins after Electrophoresis using Immunobinding with Monoclonal Antibodies," *Journal of Histochemistry and Cytochemistry*, Vol. 34, 1986, pp. 585–591.
[21] Harris, H., *The Principles of Human Biochemical Genetics*, Elsevier/North Holland Biochemical Press, New York, 1980, pp. 190–197.
[22] Budowle, B., Murch, R. S., Davidson, L. C., Gambel, A. M., and Kearney, J. J., "Subtyping Phosphoglucomutase-1 in Semen Stains and Bloodstains: A Report on the Method," *Journal of Forensic Sciences*, Vol. 31, 1986, pp. 1341–1348.
[23] Budowle, B., "Improved Separation of the Common Transferrin Variants in Gels Containing pH 5–7 Ampholines and HEPES," *Electrophoresis*, Vol. 8, No. 4, 1987, pp. 210–212.
[24] Allen, R. C., Budowle, B., Lack, P. M., and Graves, G., "Rehydrated Polyacrylamide Gels: A Comparison with Conventionally Cast Gels" in *Electrophoresis 86*, M. Dunn, Ed., VCH, Weinheim, Germany, 1986, pp. 462–472.
[25] Alexander, L. A., Reeder, D. J., and Budowle, B., "A Method for Routinely Producing High Resolution Black and White Journal Quality Photographs of Electrophoretic Gels," *Journal of Forensic Sciences*, Vol. 32, No. 5, 1987, pp. 1331–1341.

Address requests for reprints or additional information to
Bruce Budowle, Ph.D.
Forensic Science Research and Training Center
Laboratory Division
FBI Academy
Quantico, VA 22135