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Applications of Isoelectric Focusing in Forensic Serology

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ABSTRACT: The typing of certain polymorphic proteins present in human body fluids is an important aspect of the analysis of serological evidence. This is particularly true when dealing with evidence related to violent criminal activity such as homocide, assault, or rape. Until recently, the routine analysis of the genetic polymorphisms of interest relied upon conventional electrophoretic techniques such as horizontal starch or agarose slab gel or both, cellulose acetate, and vertical polyacrylamide gradient gel methods. These techniques adequately separate a limited number of common variants. In some cases, these methods are still those of choice. However, as a result of the nature of the conventional approach, problems with time required for analysis, resolution, diffusion of bands, sensitivity of protein detection, and cost are often encountered. Isoelectric focusing (IEF) offers an effective alternative to conventional electrophoresis for genetic marker typing. This method exploits the isoelectric point of allelic products rather than charge-to-mass ratio in a particular pH environment. The advantages of employing IEF include: reduction of time of analysis, increased resolution of protein bands, the possibility of subtyping existing phenotypes, increased sensitivity of detection, the counteraction of diffusion effects, and reduced cost per sample.

KEYWORDS: pathology and biology, isoelectric focusing, electrophoresis, genetic polymorphism

Biological fluids contain particular proteins which can exist in a number of alternative forms within a population. Such genetically determined systems are termed *polymorphisms* and their observable forms are called *phenotypes*. For a genetic marker system to be considered polymorphic, its most prevalent associated allele cannot occur at a frequency greater than 99% [*1*]. The identification of phenotypes of these systems is important in the determination of the genetic relationship between fluids deposited on items of evidence or at the scene of a crime and persons allegedly involved. Analyses of these markers allow for a comparison between an array of phenotypes from unknown sources and those from known specimens. Also, one can predict the probability that the particular array of phenotypes would exist in the population using the product of the probabilities of the individual phenotypes. The strength of the estimate multiplies as the number of phenotypes within each system and the number of systems increases. More importantly, these analyses can include or exclude an individual in a population that could have deposited the questioned material.

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Forensic serologists are interested in developing analytical methods for typing genetic markers in body fluids that are rapid, sensitive, highly informative, economical, reproducible, and allow for the conservation of sample. The more useful polymorphic proteins are those which exhibit a large number of alternate forms that can be readily resolved and assayed. For the past 20 years, the analyses of the majority of polymorphic systems of forensic science interest have involved the technique of *electrophoresis*. In fact, electrophoresis is used on a daily basis in hundreds of forensic science laboratories around the world. Electrophoresis employs an electric field to separate charged molecules in an environment of a particular pH. The molecule, when placed in a uniform electric field, will migrate either towards the cathode (negative pole) or anode (positive pole) at a particular rate which is dependent upon its charge character and the pH of its environment. With protein systems exhibiting multiple molecular forms, species of chemically and catalytically similar molecules will be separated as a result of slight charge-to-mass differences. Charge differences between allelic products within a genetic system are often the result of addition or removal of sialic acid residues, amination or deamination of acidic or basic amino acid protein constituents, or amino acid differences [*2,3*].

Historically, the development of electrophoretic systems for forensic science analyses has centered upon conventional techniques utilizing either cellulose acetate, starch, agarose, and to a lesser degree, polyacrylamide as a gel medium [*4*]. Conventional approaches using these media exploit the charge-to-mass properties of the molecular species of the proteins within phenotypes or chemical and structural properties or both of the media itself to effect separation. The popularity of conventional electrophoresis in the forensic science community is based upon its use and simplicity. Such systems, however, have inherent drawbacks, such as: diffusion of the sample constituents during analysis, poor resolution of protein bands, difficulty in the manipulation of the gel, electroendosmotic effects, extended analytic periods, low sensitivity, and the suboptimal methods of sample application which cause gel disturbance and foster band diffusion. The nature and extent of these problem areas support efforts within the forensic science community to seek more powerful analytical methods for the resolution of polymorphic proteins. An alternative approach for protein separation which is growing in popularity is that of *isoelectric focusing* (IEF). The sensitivity, resolving power, speed, economy, and ease of assay make IEF a desirable tool for the forensic scientist.

Background on Isoelectric Focusing

The isoelectric point (pI) of a protein is defined as the pH at which the net charge of the molecule is zero. A protein in an environment with a pH below its pI will be positively charged while its charge will be negative if the pH exceeds it pI. Thus, if a molecule is placed in a pH environment below its pI it will migrate cathodically in an electric field. If the pH environment is above its pI it will migrate anodically. When pH is equal to its pI, the protein possesses a net charge of zero and cannot migrate in the electric field. Therefore, if a polymorphic protein, with a number of species all differing in their respective pIs, is allowed to migrate in an appropriate pH gradient, its individual species could migrate to different regions of the gel because of subtle differences in their respective pIs.

The difference between the resolving capabilities of IEF and conventional electrophoresis is due to the pH environment within the gel support. In IEF, protein separation is accomplished in a pH gradient across a gel, whereas, with conventional electrophoresis, the pH is essentially constant throughout the gel. Isoelectric focusing is capable of separating proteins based upon much more subtle differences (pI) than is conventional electrophoresis (relative mobility, charge-to-mass ratio).

During IEF, a protein will migrate to the region of the gel where the pH is equal to its pI. Since its overall charge is now zero, it can no longer migrate in an electric field. Any drift or

diffusion of the protein toward the anode or cathode would result in that protein acquiring a positive or negative charge, respectively. As a result, the protein will then migrate back towards the pH zone equal to its pI. Thus, unlike conventional electrophoresis, IEF counteracts diffusion effects and concentrates proteins into sharp, stable zones. This is the reason for the superior resolution of IEF over conventional electrophoresis systems. The higher resolving power of IEF has allowed for the resolution of commonly occuring variants which were not routinely demonstrable using conventional methods. Genetic markers for which additional phenotypes ("subtypes") have been detected by IEF include phosphoglucomutase-1 [*5,6*], esterase D [*7,8*], transferrin [*9,10*], group-specific component [*11,12*], haptoglobin [*13*], and alpha 1-antitrypsin [*14,15*].

The pH gradient in IEF gels is established using synthetic compounds called "carrier ampholytes" [*16–18*]. These ampholytes are a mixture of a large number of homologs and isomers of aliphatic polyaminocarboxylic acids with different, but closely spaced, pIs. Ampholytes are commercially available in several pH ranges. Like proteins, all synthetic carrier ampholyte species have a particular pI. Therefore, each species becomes positively or negatively charged in environments with a pH below or above its pI, respectively. When an electric field is applied to the gel, the ampholytes migrate to one electrode or the other. The species with the lowest pI will migrate toward the anode (acidic electrode) and cease migration where it has assumed a zero net charge. It will then impart to the surrounding solution a pH equal to its pI. The same behavior occurs with each species in sequence from the lowest to the highest pI. Each successive species is stacked next to the one with the next lowest pI forming a pH gradient. Since diffusion of the carrier ampholytes is counteracted by the focusing effect, the gradient is stable.

For ampholytes to be suitable for IEF they must have a high buffering capacity to prevent local alteration of the pH gradient by a protein and be soluble at their respective pIs. Precipitation, which can occur with some compounds at their pI, could interrupt the current flow within the gel. Carrier ampholytes should allow for even conductivity. Low conductivity in a localized region of a gel can result in high resistance producing overheating which could denature the protein or burn the gel. In addition, high resistance causes a localized high field strength in that gel region resulting in a lower field strength over the rest of the gel to compensate for voltage differences. Thus, resolution is reduced. This effect on resolution is expected since resolution is proportional to the square root of the voltage [*19–21*]. Higher voltage gradients have been demonstrated to produce increased protein resolution [*22,25*]. In addition to the aforementioned characteristics, carrier ampholytes should not interact with proteins so as to alter their mobilities or activities or both. Finally, these synthetic compounds should have low ultraviolet absorbance at 280 nm to minimize interference with protein detection at this wavelength. An excellent review of the properties and synthesis of carrier ampholytes is given by Righetti [*18*].

Sample application in most conventional methods requires the insertion of cuttings into the gel or application of sample extracts to the gel surface in narrow zones that only partially reduces, but does not counteract, initial diffusion effects. Further, optimal protein resolution in conventional electrophoresis may be hampered by gel discontinuities necessarily imposed by cutting the gel to insert samples and by the uneven elution of proteins of interest from the supporting or substrate material. Unlike conventional electrophoresis, the effect of concentrating protein into narrow zones during IEF permits sample application to be in broad zones. Most often, samples are applied as stain extracts that have been absorbed on paper applicator tabs which are then placed on the gel surface. Cuttings from questioned stains also can be placed directly on the gel, although this is not routinely recommended because of uneven elution from the supporting material. Also, larger volumes of dilute or weak sample extracts can be applied to the gel with the expectation of IEF concentrating the proteins of interest [*26*]. Thus, some forensic science samples not previously typeable can now be analyzed using IEF.

Until recently, most IEF was performed in thin-layer slab gels (1 to 2 mm thick). Now, IEF can be performed with ultrathin-layer gels (less than 0.40 mm thick). Ultrathin gels have a greater surface area-to-volume ratio than thicker gels and, therefore, are more efficient at heat dissipation [*27,28*]. Higher voltages can be applied to these gels and an increased concentration of carrier ampholytes (4% w/v) can be used with an expectation of superior resolution [*23-25*]. In addition, the focusing of the protein bands into narrow zones results in more protein per unit volume of the gel which, in effect, increases the sensitivity of the detection of the marker system. The higher voltages also result in faster separation times making it possible to type more samples in a given amount of time than previously possible. Furthermore, by reducing the gel thickness, the quantities of reagents (and thus cost) are decreased without reducing the number of samples analyzed.

Forensic Science Applications

To date, IEF has been applied to the analysis of several genetic markers of interest in forensic serology. These systems are phosphoglucomutase-1 (PGM_1), erythrocyte acid phosphatase (EAP, AcP), transferrin (Tf), group-specific component (Gc), esterase D (EsD), alpha 1-antritypsin (Pi), and haptoglobin (Hp). In the cases of PGM, EAP, and EsD, IEF technology is already being used on a routine basis in some laboratories. For the remaining proteins, the potential for the use of IEF in forensic science analyses has either been explored on a limited basis or its implementation can be expected in the near future.

Phosphoglucomutase-1 (PGM_1)

Phosphoglucomutase-1 (PGM_1; E.C. 2.7.5.1) historically has been afforded a substantial degree of attention with regard to the separation and identification of its phenotypes by both conventional and IEF methods. This is due to its importance in both forensic bloodstain and seminal stain analysis. The analysis of PGM_1 in forensic science type materials was reported as early as 1967 [*29*]. Spencer et al. [*30*] were the first to report that a diallelic polymorphism was apparent for PGM_1. Their starch gel conventional method distinguished three common phenotypic patterns in blood samples from a large number of donors. These phenotypes were designated as "1", "2-1," and "2" based upon the four most anodal bands of activity visualized on a gel. A number of other conventional techniques have been developed which also affords the forensic serologist access to the three-phenotype array of PGM_1 [*31-37*]. Several rare variants of PGM_1 have also been identified using conventional electrophoresis [*38,39*].

Both advantages and disadvantages are encountered with the analysis of PGM_1 by conventional electrophoresis in forensic science materials. On one hand, simple methods are available that allow an analysis of PGM_1 in forensic science specimens giving limited phenotyping information using equipment that is affordable by almost every laboratory. The analysis of PGM_1 can be performed contemporaneously with the analysis of other polymorphic markers of interest such as glyoxylase I and esterase D [*37*]. Such multisystem techniques have been shown to be reliable for forensic science analyses and enjoy widespread use. These methods, however, have drawbacks typical of conventional methods of analysis. First, conventional typing methods inherently have a limited capability to distinguish among persons drawn at random from a population, based upon phenotype, because they are capable of resolving only three common phenotypes. Second, it has been our experience that conventional PGM_1 analyses of casework specimens may produce distorted patterns in which difficulty in phenotype determination, between even the two most common patterns, can be encountered. Third, the sensitivity of the conventional approach is not as great as to that of IEF [*40,41*]. This is especially important when one considers the analysis of old or diluted samples or both. Fourth, the importance of the prospect of only adequate, simultaneous analysis of

PGM_1 and other proteins may be outweighed by the increased sensitivity, resolution, and probability of discrimination obtained when utilizing the IEF method coupled with conventional methods for those markers for which the IEF method has not yet been developed.

In 1976, Bark et al. [*5*] reported that PGM_1 from erythrocytes could be resolved into ten common phenotypes when subjected to IEF. These phenotypes were designated "1+," "1−," "1−1+," "2+," "2−," "2+2−," "2+1+," "2+1−," "2−1+," and "2−1−" and resulted from the separation of the four primary bands governed by PGM_1. These results have been confirmed by Kuhnl et al. [*6*] for hemolysates, leucolysates, and sperm extracts, and by other workers for liquid and dried blood and semen, buccal cells associated with saliva, and hair root sheath cells [*42-44*]. Sutton and Burgess [*45*] have proposed classical Mendelian inheritance for the alleles governing the ten PGM_1 subtypes.

It has been demonstrated that PGM_1 analysis by subtyping methods, such as IEF, substantially improves the probability of discrimination (DP) of this system [*5*]. Therefore, one could expect to be able to differentiate between two individuals drawn at random from a population, based upon PGM_1 subtyping, at a much greater rate than with the three phenotype (conventional) analysis. The importance of the subtyping methods for PGM_1 to forensic science analyses of casework specimens is obvious.

Isoelectric focusing methodology for PGM_1 allozymes (isoenzymes coded for by individual alleles) has been developed using both agarose and polyacrylamide. Burdett [*46*] reported a thin-layer IEF method using a 1% agarose gel and pH 4 to 6.5 gradient. Several polyacrylamide approaches to PGM_1 IEF have been reported. The method of Bark et al. [*5*] used 1-mm-thick polyacrylamide gels (5.25% T) with a pH 5 to 7 gradient. Kuhnl et al. [*6*] reported the separation of PGM_1 allozymes by thin-layer polyacrylamide gel (5%T, 3%C) IEF using a pH 3.5 to 9.5 gradient and the ampholyte concentration at 2.4%. Other workers [*42-45*] have reported the use of IEF methods for PGM separation which are essentially similar to the method of Bark et al. [*5*]. The analysis of PGM_1 has also been reported using an immobilized pH 5.8 to 6.8 gradient[3].

To design an IEF system that is applicable to the analysis of forensic science samples, several considerations were taken. To minimize errors, the protein band patterns had to be linear across a gel, and the results had to be reproducible from one electrophoretic run to the next. Since the quality of case samples is often uncertain and frequently only minute amounts of sample are available for analysis, linearity, reproducibility, and sensitivity are extremely important. A highly resolving system was absolutely necessary to observe all common subtypes (or types) of this particular genetic marker system. Since many forensic serologists do not have the time and luxury to perform basic research, an IEF system had to be easy to implement in the laboratory. Also, with rising costs of laboratory equipment and supplies, the gel system had to be as economical as possible. Lastly, the use of ultrathin-layer gels was considered because of several advantages compared with thin-layer gels. Ultrathin-layer gels have a greater surface area-to-volume ratio than thicker gels and, therefore, are more effective at heat dissipation. Thus, higher voltages can be applied to the gels with the expectation of greater resolution and narrower bands. Narrower bands result in more protein per unit gel volume than broader bands, which in effect present more protein for the subsequent assay—increasing the sensitivity of detection. Furthermore, ultrathin-layer gels reduce the quantity of reagents needed to prepare the gel, and thus reduce the cost of the electrophoretic analysis.

With these considerations in mind, Budowle [*23*] developed an ultrathin-layer polyacrylamide gel isoelectric focusing (ULPAGIF) method for PGM subtyping of bloodstains that is also applicable to seminal stain analysis. To obtain the linearity across the gel and the repro-

[3]J. G. Sutton and S. A. Westwood, "Separation of the Primary Isoenzymes Bands (a−, a+, b−, b+) Determined by the PGM-1 Locus (E.C.2.7.5.1) on an Immobilized pH Gradient," Report 516, Home Office Central Research Establishment, Aldermaston, England, 1984.

ducibility of ULPAGIF runs, certain novel approaches were taken. Resolution has been shown to be dependent upon the voltage gradient [*19*]; the establishment of the pH gradient and reproducibility are also dependent upon the voltage gradient. Budowle [*23*] determined that once the ideal voltage gradient conditions were empirically determined, the voltage gradient was reproduced at intervals for every ULPAGIF run. Gels were still focused with constant power; however, the power mode was adjusted at regular intervals dependent upon the voltage parameter. By following this approach, the same results were obtained from gel to gel. To produce band linearity, it was found that the initial voltage applied to the gel during prefocusing could not exceed 250 V. As long as the conditions were reproduced, well-resolved narrowly focused PGM subtyping patterns could be obtained.

The method of Budowle [*23*] is capable of resolving all common PGM subtypes in 80 min using a 0.2-mm-thick polyacrylamide gel (5%T, 3%C) with an ampholyte concentration of 4% (w/v) and a pH 5 to 7 gradient. Since this initial report on ULPAGIF for PGM subtyping, modifications of the method have been developed to include 1.2% *N*-(2-hydroxyethyl)piperazine-*N*-3-propanesulfonic acid EPPS in the gel solution, and the use of the assay method of Divall and Ismail [*47*], which substituted meldola blue for phenazine methylsulfate. These modifications increased the sensitivity of detection of the PGM allozymes [*40*] and, with regard to EPPS, increased the separation of the 1−, 1+, 2−, and 2+ bands [*40,48*] making phenotype determinations easier. Current research in our laboratory seeks to manipulate further the migration of the PGM bands as well as enhance the sensitivity of detection of the enzyme.

During a validation study, Murch and Gambel (unpublished data) demonstrated that the subtyping of PGM by the method described by Budowle [*23*] markedly improved the rate of conclusive phenotype determinations for casework specimens. They studied a total of 2334 known liquid blood specimens, questioned dried bloodstains, and questioned dried seminal stains obtained from cases submitted to the FBI Laboratory by both ULPAGIF and conventional electrophoresis. The data revealed a 30.7% gain in conclusive PGM phenotype determinations was obtained for questioned dried bloodstains and a 11.7% gain was obtained for questioned dried seminal stains. The rate for conclusive phenotype determination for both IEF and conventional electrophoresis for known blood specimens was comparable. (The reader is reminded of the relative discriminating power of the two approaches.) These studies also revealed a 0.6% inconsistency rate between the phenotype determination obtained by these two means for all samples analyzed in this study. The inconsistencies obtained were attributed to difficulties in phenotype determinations by the conventional electrophoretic methods.

Erythrocyte Acid Phosphatase (EAP, AcP)

Erythrocyte acid phosphatase (EAP, AcP; E.C. 3.1.3.2) is a polymorphic marker of considerable forensic science importance. Its importance is due largely to the existence of six common phenotypes [*49,50*] which allow for a relatively high degree of discrimination between random individuals [*51*]. The EAP polymorphism was first described by Hopkinson et al. [*49*]. Using horizontal starch slab gel electrophoresis, they observed five phenotypes (A, BA, B, CA, CB) that differed from each other in the position and intensity of various allozymes in three primary zones of the gel. Family studies indicated that the polymorphism is governed by three codominant alleles (A, B, C), and that six common phenotypes could be expected. Several rare alleles, whose products have electrophoretic mobilities different from those of the A, B, and C alleles, have also been reported [*52–55*]. The most interesting of the less common alleles, from a forensic science perspective, is the R allele which is present at polymorphic frequencies in black populations [*52*].

Historically, starch gel electrophoresis has been the most common technique employed for EAP phenotyping of forensic bloodstain evidence. Other starch methods [*56,57*] have dif-

fered from that of Hopkinson et al. [*49*] in the buffer system used. Multisystem approaches with other proteins of interest have also been developed [*37,58*]. EAP has also been analyzed on agarose [*59*], polyacrylamide [*60*], cellulose acetate [*61*], and cellogel [*62*].

Most, if not all, conventional techniques can be expected to give inferior resolution and distinction of EAP phenotypes when compared with IEF analyses. The considerable band diffusion that occurs during conventional electrophoresis sometimes makes phenotype determinations difficult. This is especially true with the B, CB, and C phenotypes because distinction of these phenotypes is based upon the relative enzyme activity in those two regions and resultant band intensities. Any technique that minimizes diffusion would improve EAP typability. The primary C allozyme migrates only slightly anodically during conventional electrophoresis, and therefore, has substantial opportunity to diffuse. As discussed previously, conventional electrophoresis does not counteract diffusion. Slower migrating bands can be expected to diffuse to a greater degree than faster migrating ones resulting in the possible misinterpretation of true relative band intensities. Thus, a CB phenotype may appear as a B, or a C as a CB. The use of IEF methodology for EAP analysis minimizes the diffusion problems associated with conventional analysis, as well as reduces the time of analysis and avoids the necessity of having to manipulate starch gel.

A plausible forensic science approach to EAP analysis by IEF was reported by Burdett and Whitehead [*63*]. Their method used 2-mm-thick polyacrylamide gels (5% acrylamide, 0.25% bisacrylamide) with a pH 5 to 8.5 gradient. The total analytic time was approximately 3 h. Randall et al. [*64*] presented an alternative, more rapid EAP isofocusing method. Their method utilized much thinner gels (0.15 mm) and higher voltages resulting in a reduction of the run time to 75 min. They determined that the use of their technique resulted in the detection of three primary bands of activity. As with conventional electrophoresis, the distinction of phenotypes by IEF was based upon position and relative intensity of the bands. In this system, as with the method of Budowle [*24*] (see below), the "A" allozyme was the most cathodic, the "B" allozyme was the most anodic, and the "C" band was positioned between the other two. Divall [*65*] determined that IEF was a more reliable and sensitive technique than conventional starch electrophoresis for casework bloodstain analyses. A comparison of both IEF and conventional methods on 500 casework specimens indicated a 15.4% increase in conclusive phenotype determinations with IEF over the conventional method. The amount of negative determinations essentially did not change. Therefore, Divall concluded that the increase in phenotype determination was related to the resolution of those stains deemed inconclusive by conventional methods. He also indicated that approximately five times less material was required for IEF analysis compared with conventional analysis.

Since the A, B, and C allozymes were widely separated by IEF, there was no need to have an electrode wick distance of 9.5 cm. Therefore, Budowle [*24*] developed an ULPAGIF method for EAP typing on a gel with an electrode wick distance of only 5.4 cm. The approach, theory, and philosophy for this method was similar to that for PGM subtyping by Budowle [*23*], previously discussed. This technique produced linear sharp band patterns in a 30-min separation. A "cold focusing" variation of this method reduces the run time from 30 to 22 min [*24*]. A study by Murch and Budowle [*41*] determined this method to be an effective alternative to starch gel analysis of EAP in casework bloodstains. The 19.1% gain in conclusive phenotype determinations by IEF over starch electrophoresis was comparable with the data reported by Divall [*65*]. Our observations indicate that the effectiveness of IEF in forensic science analyses lies in its concentration of allozyme bands into narrow zones (counteraction of diffusion) resulting in greater accuracy in phenotype determination, its greater sensitivity, and in speed of analysis. These aspects make it important to consider IEF for EAP phenotype determinations with forensic science specimens. The method of Budowle [*24*] is employed for routine casework analysis in our laboratory. The analysis of the two other proteins (adenylate kinase, adenosine deaminase), which had been contemporaneously analyzed by the Group II starch method [*37*] in our laboratory, will be typed using an alternative, more rapid conventional method [*66*].

Esterase D

Esterase D (EsD; E.C. 3.1.1.1) is an erythrocyte-borne polymorphic enzyme. Conventional electrophoresis of EsD results in three common phenotypes (1, 2-1, and 2) which are governed by two autosomal codominant alleles, (EsD1 and EsD2). Martin [*67*] using high voltage agarose gel electrophoresis, realized that the EsD2 allele could be subdivided into an additional allele designated EsD5. The presence of the EsD5 allelic product increased the discriminating probability of EsD from 0.35 to 0.43 in whites [*68*]. Olaisen et al. [*7*] and others [*25*] reported IEF techniques capable of resolving all common phenotypes governed by the three alleles. Recently, Budowle [*25,69*] reported an ULPAGIF technique that can resolve the common variants in 53 (cold focus) to 90 min (LKB Ultrophor). This IEF approach was similar to those for PGM and EAP, previously discussed in this paper. The value of EsD, when typed by conventional means, is limited as a result of its relative instability with respect to other proteins of forensic science interest. The method [*25*] is capable of routinely detecting EsD in four-week-old bloodstains. Subtyping of EsD by IEF on casework evidence is presently being used at the Metropolitan Police Laboratory, London, England (G. Divall, personal communication).

Additional Potential Applications of IEF

The serum proteins group-specific component (Gc), transferrin (Tf), haptoglobin (Hp), and alpha-1-antitrypsin (Pi) are extremely polymorphic systems. Three of these markers—Gc, Tf, and Hp—are used for bloodstain characterization. Although the typing of these markers is performed by conventional methods in forensic science laboratories, more information can be achieved by IEF as a result of the fact that subtypes can be obtained.

Gc exists in three common phenotypes (1, 2-1, 2) when typed by conventional immunofixation agarose gel electrophoresis. Constans and Viau [*11*], using polyacrylamide gel IEF, demonstrated that the Gc1 allele can be divided into the 1F and 1S alleles. Thus, six common phenotypes were observed (1F, 1S, 2-1F, 2-1S, 1F-1S, 2). Modifications of this IEF technique have been used to subtype Gc in plasma [*12,70,71*]. Because of the increased DP achieved by subtyping Gc, there has been recent interest in applying IEF typing to Gc derived from bloodstains. Kido et al. [*72*] demonstrated that Gc in bloodstains maintained at room temperature could be subtyped up to four months. While preliminary results indicate that subtyping of Gc derived from bloodstains is feasible, one problem remains. Actin from platelets can complex with Gc, resulting in additional bands [*73,74*]. The actin-Gc complex can produce erroneous results and, therefore, limit the potential of Gc subtyping for forensic science purposes. Westwood [*75*] recently reported that 6*M* urea would break the actin-Gc complex. Using the extraction procedure described by Westwood [*75*], Budowle [*69*] was able to subtype successfully Gc derived from bloodstains using an ULPAGIF technique. This method can also resolve the 1A1 (Y, Ab) allelic product (a black variant), thereby eliminating the need for an additional test to confirm the presence of this variant.

Transferrin is currently used as a forensic science marker for bloodstain analysis, often being run simultaneously with Gc by conventional agarose gel electrophoresis. The use of conventional electrophoresis has shown the frequency of the most common Tf allele, C, to be greater than 98% for most populations [*76*]. However, Kuhnl and Spielman [*9,10*] have determined by IEF that the greatest degree of polymorphism for Tf exists in the Tf C allele. The Tf C allele can be divided into three common alleles (by IEF), C1, C2, and C3. Thus, six common phenotypes can be observed where only one phenotype was previously detectable. Budowle and Scott [*77*], Dykes et al. [*78*], and Carracedo et al. [*79*] have recently described IEF techniques followed by silver staining that produce sensitive assay systems for subtyping Tf derived from bloodstains. Budowle and Scott [*77*] demonstrated that the subtyping of Tf in six-month-old bloodstains maintained at room temperature was possible. Presently, this method is being evaluated on casework bloodstains in our laboratory.

Alpha-1 antitrypsin is the main protease inhibitor in serum. Since the first report of the genetic polymorphism of Pi [*80*], the existence of approximately 20 codominant alleles has been observed [*81*]. Allen et al. [*14*] were the first to use IEF for Pi phenotyping. This led to the identification of the M subtypes (M 1, M 2, M 3), which increased the value of Pi for genetic, paternity, and possibly forensic science studies. Carracedo and Conchiero [*82*] reported the application of IEF to the typing of Pi in bloodstains. They successfully typed Pi in bloodstains up to three months of age when stored at −4°C, up to six weeks at 4°C, and up to six weeks at 20 to 25°C. It was noted, however, that the rate of successful typing varied dramatically when other environmental conditions were imposed upon the bloodstains. Unfortunately, their method could not subtype the M allelic products, thus missing a high degree of genetic information. Recently, Budowle and Murch [*83*] have developed a highly resolving, rapid IEF method for subtyping Pi that is presently being evaluated for use in bloodstain analysis. In addition, we are investigating the potential of Pi for the analysis of sexual assault evidence, since its presence in semen has been indicated [*84*].

Haptoglobin is a serum glycoprotein of the alpha 2-globulin class which binds hemoglobin that is released into the vascular circulation. As a forensic science marker, Hp is extremely valuable because it is polymorphic [*85*] and very stable in bloodstains [*86*]. In most laboratories, conventional electrophoresis (starch gel, gradient polyacrylamide gel, and discontinuous polyacrylamide gel) is used to detect three common phenotypes (1, 2-1, 2). While these methods are reliable, they do not permit the subtyping of the Hp alpha chain. Teige et al. [*13*] have developed an IEF method for Hp subtyping that does not require prior purification of serum samples. At least eight different phenotypes for Hp were observed. This method is presently under investigation in our laboratory for bloodstain analysis.

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