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A Double Origin Electrophoretic Method for the Simultaneous Separation of Adenosine Deaminase, Adenylate Kinase, and Carbonic Anhydrase II

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ABSTRACT: A rapid, reliable method for the simultaneous separation of adenosine deaminase, adenylate kinase, and carbonic anhydrase II by agarose gel electrophoresis is presented. This method uses a double origin sample application system. Unreduced sample extracts for adenylate kinase analysis are applied 13.0 cm from the anode. Reduced sample extracts for the remaining proteins of interest are applied 7.0 cm from the anode. The use of applicator foils and an increased voltage gradient result in superior resolution, linearity, and band sharpness of the allozyme patterns. Further, there is no masking of the adenylate kinase 2 band as a result of the use of a reducing agent, and carbonic anhydrase II is resolved without interference from hemoglobin as has been observed with other multisystem methods.

KEYWORDS: forensic science, adenosine deaminase, adenylate kinase, carbonic anhydrase, electrophoresis, agarose gel, allozyme, double origin

Adenosine deaminase (ADA; E.C. 3.5.4.4), adenylate kinase (AK; E.C. 2.7.4.3), and carbonic anhydrase II (CA; E.C. 4.2.1.1) are polymorphic proteins of interest in the typing of both questioned and known forensic blood specimens. Common variants of these proteins have been separated by a variety of methods utilizing such supporting media as starch [*1-3*], agarose [*4*], polyacrylamide [*5,6*], and cellulose acetate [*7-9*]. For several years, our laboratory employed the Group II multisystem method [*10*] for the simultaneous separation of ADA, AK, and erythrocyte acid phosphatase (EAP, AcP); and the Group IV multisystem method [*11*] which includes the analysis of CA II. The results obtained in thousands of casework analyses, by using these methods, however, encouraged the development of alternative approaches to the separation of these markers.

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Recently, our laboratory implemented a more rapid, reliable, sensitive, and accurate EAP typing method [*12,13*] which supplanted the traditional starch method. Therefore, an alternative method for ADA and AK analysis was sought. This method had to overcome problems associated with the Group II, Group IV, and other multisystem methods relating to: time of analysis, resolution and sharpness of allozyme bands, effects of reducing agents on the masking of the AK 2 band, interference of hemoglobin with the determination of common CA II variants, and the practice of cutting slots in the gel for sample application which may promote band distortion [*14*]. It was determined that, because of the low probability of discrimination of each of these markers, the use of single system methods for each protein was unwarranted.

We report here the development of a new method for ADA, AK, and CA II electrophoresis, termed the "double origin" (DO) method, which overcomes the aforementioned problem areas of other systems by the use of: (1) an agarose gel with two separate sample application zones; (2) separate extractants for ADA/CA II and AK; (3) applicator foils for the presentation of sample extracts to the gel; and (4) an increased voltage gradient resulting in a run time of only 90 min.

Methods and Procedures

Samples

Dried bloodstains ($N = 93$) were prepared on washed cotton sheeting from known casework liquid blood specimens submitted to the FBI Serology Unit. Standard ADA, AK, and CA II phenotypes were obtained from donors in the FBI Laboratory by finger puncture and were collected on clean cotton thread. All 93 known specimens and the standards were typed by both the DO method and by the Group II and Group IV methods [*10,11*]. Additionally, 200 known and 696 questioned casework samples (submitted to the FBI Laboratory for routine analysis) were compared with previously typed standards by the DO method.

Gel Preparation

One percent agarose gels (Type I, Sigma; 1.0 mm thick) were cast on untreated, clear glass plates of dimensions 18.5 by 21.0 cm. The gel buffer was 5.7m*M* sodium phosphate, dibasic (Na_2HPO_4): 2.50m*M* citric acid, pH 5.5 [*10*]. The surface of the gel was blotted with Whatman No. 1 paper before sample application.

Sample Preparation and Application

Two cuttings (0.5-cm^2 area or 1.5-cm thread length) from each sample were extracted for 45 min at room temperature, one in 20 μL of distilled water (AK analysis) and the other in 20 μL of 50m*M* 2-mercaptoethanol (Sigma; ADA/CA II analysis). Samples were applied to the gels as extracts using two applicator foils (LKB) per plate. The first foil was applied 7.0 cm from the anode end of the plate. The other foil was placed 13.0 cm from the anode end of the plate parallel to the first. For each AK extract, 7 μL was applied to individual wells of the 13.0-cm foil, and 7 μL of each ADA/CA II extract was applied similarly to the 7.0-cm foil. The extracts were allowed to absorb into the gel for 10 min. Each foil was then blotted and removed. The positioning of the origins and the respective areas of subsequent assay for each protein are shown in Fig. 1.

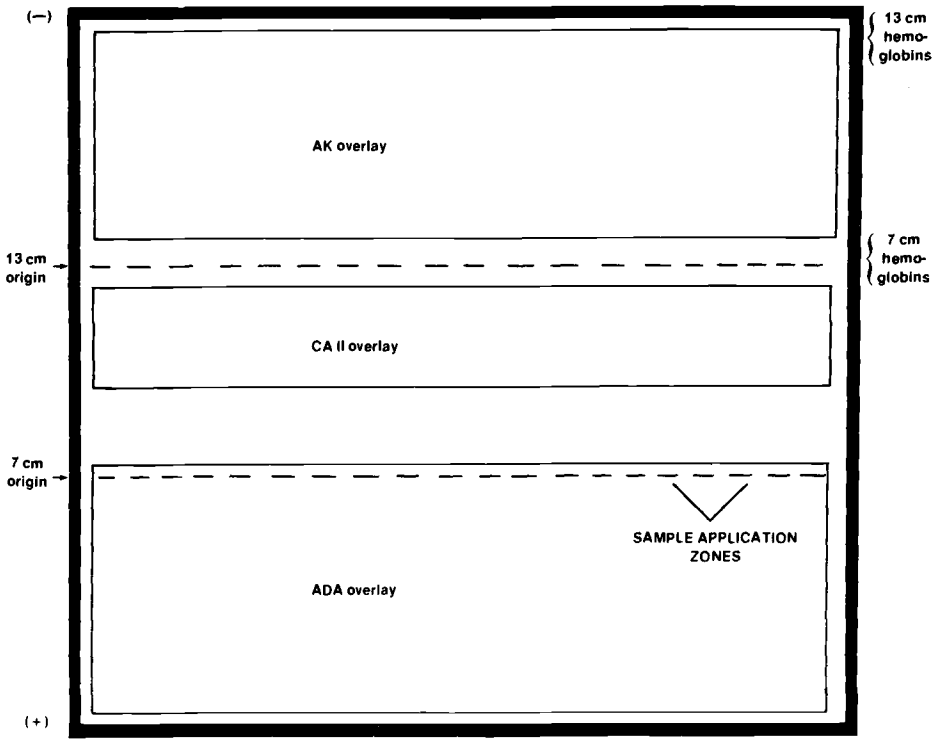

FIG. 1—*Arrangement of sample application origins and assay overlay zones for the double origin method.*

Electrophoresis

Electrophoresis was performed using a Vokam Model 500-500 power supply (Shandon Catalog No. 541 × 52) and Shandon electrophoresis tanks (Shandon Catalog No. 600 × 100) with cooling supplied to the platforms by a circulating bath (Model 2006, Forma Scientific) set at 4°C. The wicks were constructed of three layers of Whatman No. 3 paper with a inter-wick distance of 16.5 cm. The tank buffer was 0.29*M* Na_2HPO_4:0.10*M* citric acid, pH 5.5 [*10*]. The running conditions were 350 V (approximately 60 mA) for 90 min.

Assay

Following electrophoresis, CA II was assayed for by overlaying the area from the hemoglobin line to 4 cm anodic with Whatman No. 1 paper that had been soaked with a solution of flourescein diacetate (4 mg/10 mL of 0.2*M* NaH_2PO_4:0.09*M* Na_2HPO_4, pH 6.2) [*11*]. The ADA and AK assays were performed according to the Group II procedure [*10*]. The AK overlay was applied from the hemoglobin line to the cathode end of the plate and the ADA overlay was applied from the 7.0-cm origin to the anode end of the plate, using preformed molds. The plates were then incubated at 37°C in the dark. The results for CA II were read after 15 min under long-wave ultraviolet light. The results for ADA and AK were read after an additional 30-min incubation at 37°C using transmitted flourescent light.

Results

Typical electrophoresis patterns of samples analyzed for the three proteins of interest by the DO method are shown in Figs. 2, 3, and 4. This method consistently produced linear, reproducible patterns across the gel for all three proteins. With the DO method, CA II migrated cathodically from the 7.0-cm origin at a slower rate than its accompanying hemoglobin and thus was easily detected in the center of the plate. Adenylate kinase migrated cathodically from both sets of samples but was assayed for only in the region cathodic to the

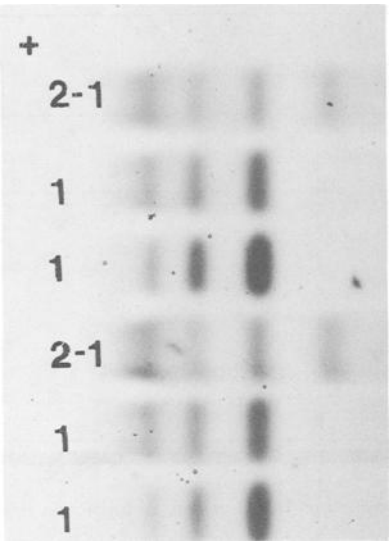

FIG. 2—*ADA allozyme banding patterns by the double origin method.*

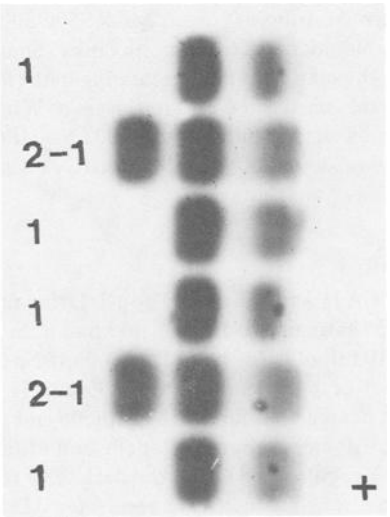

FIG. 3—*AK allozyme banding patterns by the double origin method.*

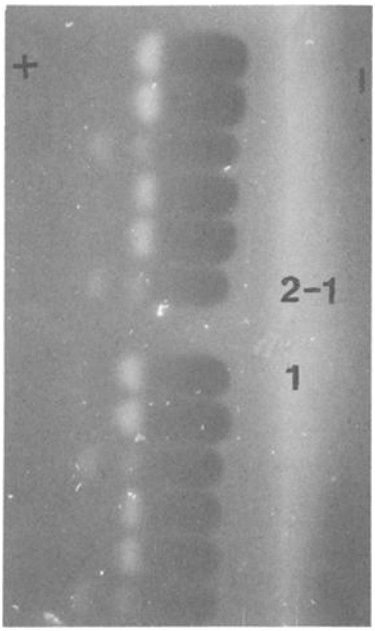

FIG. 4—*CA II allozyme banding patterns by the double origin method.*

13.0-cm origin. Adenosine deaminase migrated anodically in a similar fashion but was assayed for only in the region anodic to the 7.0-cm origin.

For comparative purposes, 93 known liquid blood specimens from cases submitted to the FBI Laboratory were typed by both the DO and applicable "Group" method. The data obtained from this study are presented in Table 1. No inconsistencies were observed with regard to the phenotype patterns obtained for each sample (when conclusive) analyzed by DO

TABLE 1—*A comparison of typing efficiency of the double-origin method and two other multisystems for ADA, AK, and CA II typing.*[a]

System	No.	Conclusive (%)	Inconclusive (%)
		GROUP II	
ADA	93	79 (84.9)	14 (15.1)
AK	93	90 (96.8)	3 (3.2)
		GROUP IV	
CA II	93	82 (88.2)	11 (11.8)
		DOUBLE ORIGIN	
ADA	93	83 (89.3)	10 (10.7)
AK	93	92 (98.9)	1 (1.1)
CA II	93	87 (93.6)	6 (6.4)

[a]Percent increase in typing efficiency, double origin versus either Group II or Group IV: ADA, 4.4; AK 2.1; and CA II, 5.4.

TABLE 2—*ADA, AK, and CA II analysis of forensic specimens by the double-origin method.*

System	Sample Type	No.	Conclusive (%)	Inconclusive[a] (%)
ADA	known	200	139 (69.5)	61 (30.5)
	questioned	696	338 (48.6)	358 (51.4)
AK	known	200	183 (91.3)	17 (8.7)
	questioned	696	491 (70.6)	205 (29.4)
CA II	known	200	144 (72.0)	56 (28.0)
	questioned	696	212 (30.4)	484 (69.6)

[a]Includes both inconclusive and negative activity determinations.

and "Group" approaches or when compared with the standards. The data in Table 1 indicate that the typing efficiency of the DO method may be slightly greater (4.4% for ADA, 2.1% for AK, 5.4% for CA II) than the applicable "Group" method for each of the proteins of interest. The data obtained from the analysis of the 896 additional forensic specimens by the DO method are presented in Table 2. No inconsistencies occurred between allozyme banding patterns obtained for these three markers and the previously typed standard phenotypes when conclusive determinations could be made. The percent typing efficiency of the DO method for ADA, AK, and CA II for known and questioned specimens for this sample population was 69.5/48.6, 91.3/70.6, and 72.0/30.4, respectively.

Discussion

This communication presents a new rapid, reliable method for the phenotypic analysis of ADA, AK, and CA II in agarose gels. This method appears to have several advantages over previously reported techniques. Well-defined, linear, easily discernable allozyme banding patterns are obtained for all three markers. This is in contrast to other multisystem methods [*10,11*] in our experience. The DO method achieves these results by the application of the samples as extracts in narrow zones using sample applicator foils and employing, by conventional electrophoretic standards, a relatively high field strength (21.2 V/cm). The use of a relatively high field strength allows for the separation of the bands of interest in a short period of time (90 min) with the minimization of band diffusion, which occurs in conventional systems employing low voltages and long running times. By using the applicator foils, samples are applied in very narrow zones on the surface of the gel, restricting the area of sample contact with the gel, and are allowed to diffuse into the gel minimizing any alteration of gel integrity. Budowle [*14*] has noted that the practice of cutting slots in a gel and placing foreign materials in it may induce subsequent band distortions during protein separations. Also, when samples are applied as extracts rather than on materials embedded in a gel, the potential for uneven elution of proteins from the substratum is eliminated. Further, band linearity and definition are enhanced in the DO method because of the elimination of starch from the gel system. Starch is not as consistent in properties for electrophoresis from lot to lot compared with agarose (B. Budowle, personal communication). With this in mind, an agarose system can be expected to be more reproducible and manipulable than a comparable starch system.

Another aspect of the DO method that results in improved patterns, especially for AK, is the use of a separate extractant for the portion of the sample to be subjected to ADA/CA II analysis versus that which is to be analyzed for AK. By using paired extracts for each specimen to be analyzed, that which is to be analyzed for ADA can be properly reduced while the AK phenotype is free from interference of the reducing agent. This is also encouraged by

preventing contact between the reducing agent and the region of the gel to be assayed for AK. Detrimental effects of reducing agents have been noted in a single origin, 90-min ADA/AK technique (C. Barrera, personal communication). Unlike other multisystems, the detection of CA II phenotypes is made easier because of the linearity and sharpness of the banding patterns. Also, hemoglobin does not migrate to the same region of the gel as does CA II thus eliminating the quenching of allozyme band fluorescence.

The method presented herein has been shown to be reliable for the typing of ADA, AK, and CA II in forensic specimens. The data given in Table 1 suggest that a slight increase in typing efficiency for these three proteins can be obtained by the use of the DO method. This is of some additional value particularly when one considers the reduced time of analysis. The data in Table 2 reflect the typing efficiency of the DO method for a larger sample population consisting of both known and questioned specimens. It is pointed out that differences occurred in absolute typing efficiency for known specimens typed by the DO method between these two studies. This may be attributable to the sample population studied, as typing efficiency is highly dependent upon the quality and condition of the samples submitted for analysis. It is difficult for our laboratory to control these aspects. Further, a comparative study using both the DO and "Group" methods was not performed on either the 200 known or 696 questioned specimens in the second population. Thus, its comparative efficiency on a sample-by-sample basis in the larger group cannot be ascertained. Inspection of data from another study conducted in our laboratory, related to the typing efficiency of the Group II and Group IV systems for ADA, AK, and CA II in casework specimens, indicates that the success rates are comparable to that obtained by the DO method. Again, no inconsistencies in allozyme patterns were obtained between the DO and "Group" methods when a conclusive determination could be made. The value of this new method appears to lie in its ability to resolve and sharpen the allozyme bands for each protein in and the speed of analysis.

The DO method is currently being used in our laboratory for routine forensic science analyses of casework specimens in conjunction with a rapid, reliable isoelectric focusing method for EAP [*12*]. This allows for the rapid and effective analysis of the traditional Group II proteins plus CA II.

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