J Forensic Sci, Jul. 1977, Vol. 22, No. 3

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Haptoglobin Phenotyping of Bloodstains by Nongradient Polyacrylamide Electrophoresis

Haptoglobin is a plasma glycoprotein with α_2 electrophoretic mobility which plays a role in hemoglobin catabolism. It is of interest to the forensic scientist because it exhibits polymorphism. The three most common phenotypes are designated Hp 2-1 (the heterozygous state), Hp 2-2, and Hp 1-1.

Haptoglobin typing of bloodstains has been accomplished by starch block electrophoresis [*1*], immunoelectrophoresis [*2*], continuous gradient polyacrylamide gel electrophoresis [*3,4*], and step gradient polyacrylamide gel electrophoresis [*5*].

This paper presents a simplified technique for haptoglobin typing using a 6% nongradient (homogenous) slab of polyacrylamide gel supported on a glass plate. The technique allows successful phenotyping of a single bloodstained thread. Additionally, an aqueous glycerin wash preserves the developed plate.

Materials and Methods

Apparatus

For this investigation a water-cooled electrophoresis system based on the design of Laurell and Nilehn [*6*] was used. This is available from MRA Corp., Boston, Mass., as Standard Apparatus #92-1. The glass plates [4.25 by 8-in. (108 by 203-mm), single-strength window glass] were obtained from Independent Glass Co., Brookline, Mass.

Chemicals

Diethyl barbituric acid and sodium diethyl barbiturate were obtained from Sigma. Calcium lactate was from Matheson, Coleman and Bell. Acrylamide; N,N-*bis*-methylenediacrylamide; N,N,N′,N′-tetraethylmethylenediamine; ammonium persulfate; and *o*-tolidine[4] were from Eastman. Acetic acid, sodium acetate, and hydrogen peroxide (30%) were from Fisher. Diethylene glycol and 4-chloro-1-naphthol were from Aldrich, while agarose was obtained from Miles. All chemicals were reagent grade.

Received for publication 2 Sept. 1976; revised manuscript received 13 Dec. 1976; accepted for publication 20 Dec. 1976.

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[4] A less carcinogenic chromogen (3,5,3′,5′-tetramethyl benzidine) than *o*-tolidine has been reported [*7*].

Electrophoresis Wicks

In the electrophoretic apparatus, electrical contact was made between the buffer in each vessel and the separating gel by two agarose wicks, one vertical and one horizontal. The vertical wick was formed in the cooling support, while the horizontal one was preformed in a mold and applied to the chamber after the electrophoresis gel plate was in place. The horizontal wick traversed the gap between the vertical wick and the gel.

These wicks were made of agarose (1.5% in buffer). The horizontal wicks were cast between two clean uncoated glass plates separated by a $\frac{1}{16}$-in. (1.6-mm) thick plastic spacer. The agarose solution was heated to about 60 °C, then poured into the mold. After cooling to 4 °C, the glass plates were separated and wicks 1.5 and 2.5 cm wide were cut with a spatula. These wicks were stored in a tray of buffer at 4 °C, which enhanced their durability. After each run, the buffer was changed and the wicks were saved. The wicks could be reused as many as twelve times. The vertical wicks were cast directly in the cooling support according to the manufacturer's instructions.

Buffer

The buffer used throughout this work was diethyl barbital, pH 8.6 (0.05*M*), containing calcium lactate (0.0018*M*). Sodium diethyl barbiturate (70.1 g), diethyl barbituric acid (11.0 g), and calcium lactate pentahydrate (4.6 g) were dissolved in distilled water (about 7 litres) with the aid of a magnetic stirrer. The pH was measured, and if necessary, adjusted to 8.60 ± 0.05 with 1*N* hydrochloric acid. Sufficient water was then added to give a total volume of 8 litres.

Stain

The stain used to visualize hemoglobin and hemoglobin-haptoglobin complexes was always prepared just before use. Eighty millilitres of 7% aqueous acetic acid (7 ml of concentrated acetic acid per 100 ml of solution) and 80 ml of 1*M* sodium acetate were combined in a 250-ml Erlenmeyer flask. To this was added, with constant swirling, 1 ml of 2% (w/v) 4-chloro-1-naphthol in diethylene glycol, 5 ml of saturated *o*-tolidine in 7% acetic acid, and 5 ml of 3% aqueous hydrogen peroxide.

Nongradient Polyacrylamide Gel

The glass plates used to support the thin-layer polyacrylamide gels were washed in warm laboratory detergent and then rinsed three times in warm tap water and three times in distilled water. After air drying, one side of the plate was washed with a large cotton swab soaked in methanol. The plate was laid on a level surface, and, after the methanol had evaporated, approximately 8 ml of an agarose solution (0.3% in distilled water heated to about 70 °C) was spread over each plate. The agarose layer was made uniform by drawing a pipet in contact with the agarose horizontally over the plate, first slowly to one edge of the plate and then smoothly across to the other edge. The plates were dried with a stream of warm air. The agarose-coated side was marked with a diamond stylus and the plates were stacked and stored at room temperature until needed.

A solid mixture of acrylamide and N,N-*bis*-methylenediacrylamide (95:5 by weight) was prepared. This mixture was stored dessicated in the refrigerator. Thin-layer gels were made by swirling the acrylamide mixture (2.4 g), barbital buffer (40 ml), and N,N,N′, N′-tetraethylenediamine in a beaker until solution was complete. Sufficient ammonium persulfate (about 35 mg) was added with swirling so that gel formation would occur in about 5 min. After 20 s of swirling, the solution was pipeted into a gel mold composed in succes-

sion of an agarose-coated glass plate, a 1/32-in. (0.8-mm) plastic spacer and a plastic, slot-forming plate [*8,9*]. After 10 min, the plastic plate and spacer were carefully removed, leaving a thin layer of gel bonded to the agarose-coated plate. The samples were applied, and the electrophoresis was performed immediately. Care was taken to minimize exposure of the gel to air, as it dries out rapidly.

Samples

Bloodstains were prepared by dropping fresh blood (0.2 ml) onto cotton fabric. The fabric was exposed to the air at 25 °C for 3 days and then kept in sealed tubes at 4 °C. After clotting for 1 h, the rest of each blood sample was centrifuged and the supernatant serum was removed and stored at 4 °C.

Hemolysate

The hemolysate was prepared from blood samples drawn into ethylenediaminetetraacetic acid (EDTA). After centrifugation and removal of the plasma, the cells were washed three times with physiological saline. One volume of cells was then lysed with one volume of distilled water and 0.25 volume of toluene. This mixture was shaken vigorously and frozen. Upon thawing, the mixture was centrifuged for 10 min, and the aqueous layer filtered through Whatman #2 filter paper. This filtrate was diluted 1:5 with buffer, divided into 1-ml aliquots, and frozen. These hemolysates were used for a least three months.

Sample Preparation

Serum (100 μl) was mixed with hemolysate (50 μl) in an 8 by 75-mm test tube. The tube was sealed with Parafilm® and incubated at 37 °C for 15 min immediately before the electrophoresis.

For the bloodstain analysis, a thread (about 2 cm in length) was teased out of the bloodstained area of a cloth with a forceps. The thread was placed in a test tube (8 by 75 mm), 50 μl of buffer was added, and the tube was sealed with Parafilm and incubated at 37 °C for 15 min immediately before the electrophoresis.

Electrophoresis

Approximately 7 μl of sample was introduced with a capillary tube into each well cast in the thin-layer polyacrylamide gel. The gel plate was placed in the electrophoresis apparatus (Fig. 1) and the horizontal wicks were positioned to overlap the vertical wicks and the gel slab. The chamber was covered with a glass cover plate, and electrophoresis was performed with cooling (4 °C circulating water) for 3 h at 175 V. These conditions moved the free hemoglobin band from the origin (sample well) to the edge of the anodic horizontal wick, that is, about 7 cm.

After immersion for 20 min in 170 ml of freshly prepared staining solution, the plate was washed and desalted in three successive baths of cool tap water (200 ml) for 15 min each, and then for 1 h in a bath of 200 ml of water containing 2 ml of glycerin. Air drying at room temperature for 48 h converted the gel into a permanent, thin film which adhered firmly to the plate and showed all of the staining details. If the glycerin bath was omitted, the film frequently peeled off the plate within two days. Glycerin concentrations higher than 1.0% (v/v) either produced a gel which remained tacky for several days (for example, 1.5% glycerin) or for much longer periods (for example, 2.0% or more glycerin).

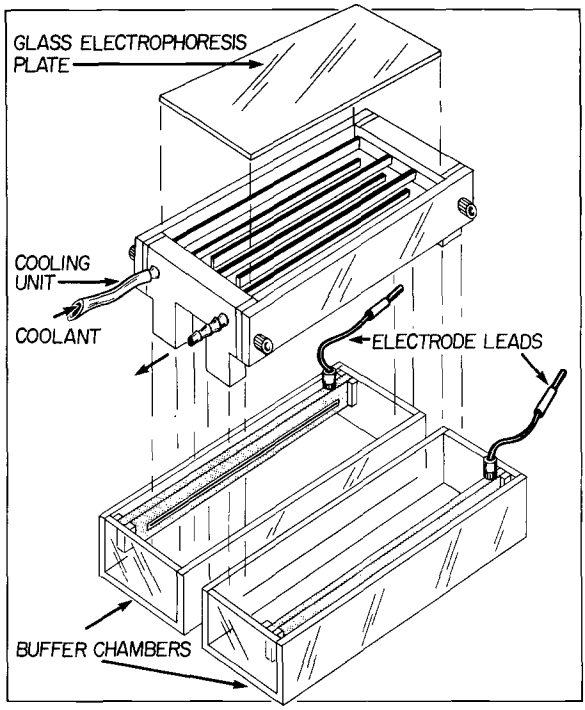

FIG. 1—*Electrophoresis apparatus and gel plate.*

Results and Discussion

Haptoglobin has received much attention in regard to bloodstain analysis for several reasons. It occurs as three rather common phenotypes, is present in reasonably high concentration in bloodstains, has reasonable stability in such samples, and can be readily visualized after electrophoretic separation because of its ability to form a highly stainable complex with hemoglobin. The distribution of the three common phenotypes 1-1, 2-1, and 2-2 in the white population of the United States has been reported to be 13.2, 50.6, and 36.2%, respectively [*10*].

Currently used methods for haptoglobin typing are effective but subject to certain shortcomings. For example, nongradient starch gel is used, but this medium sometimes is difficult to control. Polyacrylamide gel is more reliable, but only gradient polyacrylamide methods have been reported in the forensic literature for haptoglobin typing. Gradient generation, of course, requires extra work and equipment.

This paper reports a nongradient polyacrylamide method for haptoglobin phenotyping. The results obtained with blanks and serum samples are shown in Fig. 2*a*. A blank sample of buffer underwent electrophoresis in Slots 1, 5, and 9; a Type 1-1 serum was in Slots 2 and 6; a Type 2-1 serum was in Slots 3 and 7; and a Type 2-2 serum was in Slots 4 and 8. All samples contained added hemoglobin. The patterns are readily differentiated and correspond quite closely to those for haptoglobin typing with gradient polyacrylamide reported previously [*3,5*]. Haptoglobin 1-1, being monomeric, migrates as a single band about halfway up the gel layer. Haptoglobin 2-2 migrates as a series of bands, all within a short distance of the application trough, because this phenotype occurs as a mixture of polymers. Haptoglobin 2-1 has the general appearance of a mixture of the 1-1 and 2-2 phenotypes except for some differences in band positions and intensities.

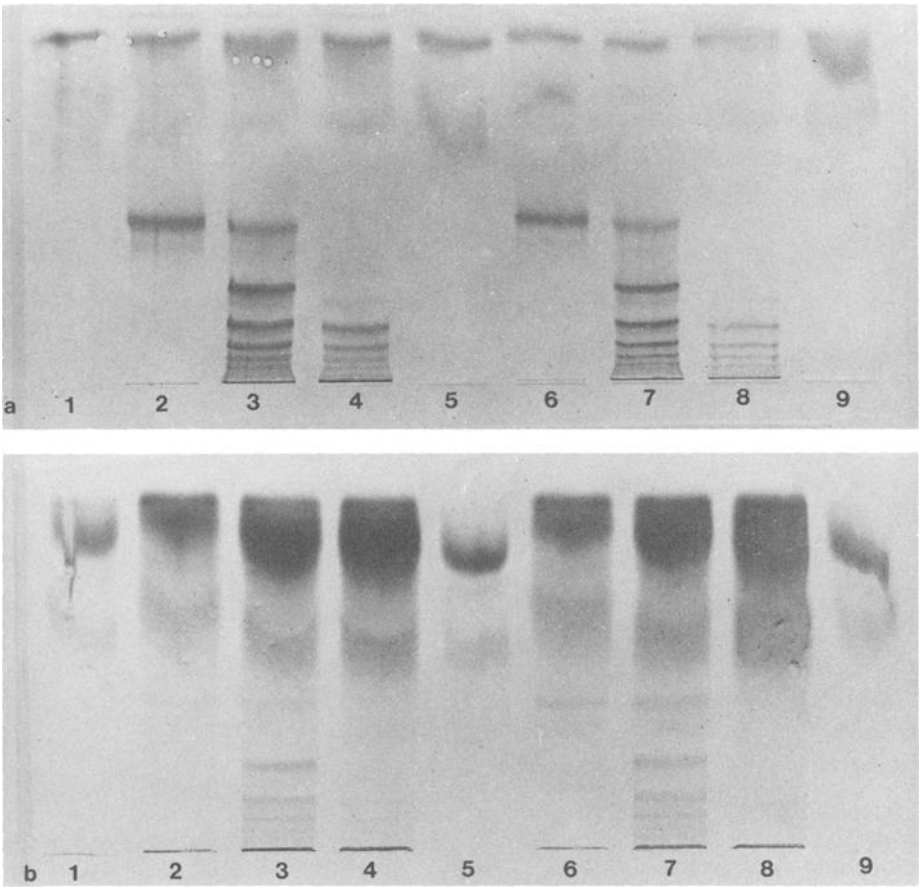

FIG. 2—*Electrophoresis patterns obtained from corresponding* (a) *serum samples and* (b) *bloodstain extracts. All samples in* (a) *were treated with hemoglobin before the electrophoresis. Patterns 1, 5, and 9 are from buffer; Patterns 2 and 6 from haptoglobin 1-1, Patterns 3 and 7 from haptoglobin 2-1, and Patterns 4 and 8 are from haptoglobin 2-2.*

The ability to provide a sensitive analysis of typical samples was demonstrated by applying the technique to single threads from one-month-old bloodstains. The results are shown in Fig. 2*b*. The samples and blanks are basically the same as those shown in corresponding slots in Fig. 2*a* except that extracts of bloodstained threads subjected to electrophoresis are shown in Fig. 2*b* instead of sera. Although the haptoglobin patterns are weaker in intensity than those obtained from the serum samples, they are still fully defined.

Thus, a nongradient polyacrylamide method for haptoglobin typing is presented and its usefulness in forensic studies is demonstrated. Treatment of the electrophoresis gel plate with an aqueous glycerin solution provides a permanent record of the analysis.

Acknowledgment

We wish to thank Dr. Chester Alper, Director of the Center for Blood Research in

Boston, for generously giving us many of the procedural details that are reported in this paper. This work was supported in part by the Law Enforcement Assistance Administration, U.S. Department of Justice, under an educational development grant, and in part by Grant RR07143, Department of Health, Education and Welfare, given to Northeastern University.

Contribution 17 from Northeastern University, Institute of Chemical Analysis, Applications and Forensic Science, Boston, Mass.

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