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An Efficient Method to Eliminate Streaking in the Electrophoretic Analysis of Haptoglobin in Bloodstains

Haptoglobin is an α_2 glycoprotein, found in the plasma portion of blood, whose biological functions include the binding and transportation of hemoglobin. Haptoglobin possesses the two characteristics necessary for it to be of value to forensic serology. First, haptoglobin is a genetic marker with an excellent "Discriminating Power" [*1*] of 0.60 [*2*], making it, mathematically, one of the best polymorphic proteins for individualizing bloodstains. Second, haptoglobin has been shown to persist in quantities readily detectable in dried bloodstains over a longer period of time than that commonly acknowledged for many other polymorphic proteins and enzymes. During the course of this study, bloodstains stored at room temperature in the laboratory for up to two years were correctly phenotyped in haptoglobin by the procedure presented here.

Despite the superior stability it exhibits in dried bloodstains, haptoglobin has not gained wide acceptance by forensic science laboratories as a genetic marker in the routine analysis of bloodstain evidence. Partly to blame has been the alleged requirement for specialized vertical gel electrophoresis equipment frequently described in the literature on haptoglobin. Despite the existence of methods for haptoglobin analysis that employ common horizontal electrophoresis equipment [*3–5*], many forensic science laboratories do not conduct haptoglobin analysis simply because of the lack of vertical gel electrophoresis equipment. Also to blame, perhaps, is the fact that haptoglobin electrophoretograms from bloodstains older than several weeks are frequently masked by trailing dark streaks caused by the products of hemoglobin deterioration[3] in the bloodstains.

Numerous methods have been published describing techniques to remove or avoid the unwanted products of hemoglobin deterioration. Blake and Sensabaugh [*6*] offer a method of sodium dodecyl sulfate (SDS) gel electrophoresis that requires the use of monospecific human haptoglobin antiserum to isolate haptoglobin from the liquid extract of a bloodstain

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[3]As it refers to the hemoglobin present in bloodstains, the term "deterioration" is used to denote any alteration of the structure of hemoglobin brought about by the normal effects of aging. The molecular modifications of hemoglobin may include the formation of aggregates, polymers, and derivatives as well as the breakdown products of decomposition.

by immunoprecipitation. Gazaway [7] describes an acid extraction involving disc electrophoresis at a pH near the isoelectric point of the haptoglobin-hemoglobin complex, followed by the manual withdrawal of the liquid samples from the tubes containing the first gels and ultimately depositing the liquid samples onto a second gel for additional electrophoresis. Of course, the classical biochemical techniques of gel filtration and column chromatography have also been used in the removal of unwanted hemoglobin and the products of hemoglobin deterioration [8].

A simpler method was sought. In the course of this study a straightforward chloroform extraction procedure was developed; it entirely eliminates the streaking effect of deteriorated hemoglobin from haptoglobin electrophoretograms. Moreover, the chloroform technique requires substantially less physical manipulation of the samples than do other methods.

Background

Chloroform has the capacity to precipitate either hemoglobin or its derivatives from certain aqueous solutions, depending on the pH, temperature, ionic strength, and dielectric constant of the solution. The chloroform extraction method, as applied to liquid blood, is a well-known and venerable biochemical procedure dating back at least to the turn of the century [9,10].

For more than the past decade, Tashian and his co-workers [11,12] have employed a chloroform and ethanol extraction technique for the removal of hemoglobin from whole blood in the purification of erythrocyte carbonic anhydrases. The method Tashian reports is an adaptation of a chloroform and ethanol extraction procedure[4] that has been traced as far back as the work of Tsuchihashi [13], reported in 1923. Tsuchihashi cited the published work of Krüger [9] as an early reference for the chloroform extraction method. From Krüger's account of 1901, it is apparent there existed some question as to who deserved proper credit for discovering the phenomenon of hemoglobin precipitation by chloroform. More recently, a bloodstain extraction method using chloroform for the analysis of Group-specific Component (Gc) was reported by Zajac [14].

The true origin of the chloroform extraction technique may be buried in antiquity, but the value of its application to haptoglobin analysis of bloodstains is indeed significant.

Materials and Methods

In this study the analysis of haptoglobin was performed by polyacrylamide gel electrophoresis as described by Culliford [15], with a Gradipore® vertical gel electrophoresis tank (Model GS-425, Isolab, Inc., Akron, Ohio) and 4 to 20% density gradient polyacrylamide gels prepared in the laboratory. However, the chloroform extraction procedure is equally applicable to any method of vertical or horizontal electrophoresis employing either density gradient or homogeneous gels.

Sample Preparation

From bloodstained fabric, cut a portion approximately 3 mm square. In the case of blood crust, approximately 1 mg is normally sufficient. Place the samples in microcentrifuge tubes

[4]Ethanol was included in their procedure to insure the removal of free hemoglobin as well as the derivatives of hemoglobin. Inasmuch as free hemoglobin itself causes no interference in the routine phenotyping of haptoglobin by electrophoresis (normal hemoglobin, Hb A, constitutes a single band of protein that migrates anodically relative to the haptoglobin bands), the removal of free hemoglobin was of no particular consequence to this study. It was found that chloroform, by itself, is capable of removing those products of hemoglobin deterioration responsible for obscuring the haptoglobin electrophoretograms. The addition of ethanol proved to be superfluous.

(the 400-μl disposable plastic tubes used in this study were Model MC-1 microcentrifuge tubes, Cole Scientific, Calabasas, Calif.).

To each sample add approximately 40 μl of an aqueous solution of sucrose, 10% (w/v). The sucrose provides an increase in the density of the liquid samples necessary for their application into the sample wells of vertical gels; however, for horizontal gel methods, distilled water may be substituted.

Cap the tubes and allow the stains to dissolve at room temperature for a minimum of 1h. In the case of stains older than several months, it may be necessary to allow them to dissolve overnight.

Extraction Procedure

Remove the portions of fabric from the microcentrifuge tubes by using a conventional wooden applicator stick to squeeze as much residual liquid as possible from the fabric by pressing it against the side of the tube with the applicator stick.

Add approximately 200 μl of chloroform to each tube and shake vigorously by hand (or with a mechanical shaker) for 1 to 2 min. Centrifuge sufficiently to separate the liquid phases. A thin wafer of reddish-brown precipitated matter containing the products of hemoglobin deterioration will appear at the interface, separating the aqueous phase above from the chloroform phase below.

Collect the aqueous supernatant with a double-drawn pipet, exercising care not to disturb the insoluble material at the interface, and apply the liquid sample directly to the gel. Proceed with electrophoresis.

Experimental Procedure

To compare the electrophoresis results of bloodstains extracted by the chloroform method with those not subjected to chloroform extraction, the following experimental procedure was employed.

Bloodstains were produced under laboratory conditions from fresh whole blood (with no chemical additives) on clean wool fabric and on cotton fabric. The bloodstains, ranging in age from several weeks to several years, were extracted with a 10% sucrose solution by the method described previously. Each bloodstain solution was then divided into two equal volumes: one aliquot was extracted with chloroform as outlined above, and the other remained unextracted.

The paired sets of extracted and unextracted bloodstain solutions were subsequently applied to adjacent wells in polyacrylamide gels and subjected to electrophoresis. Development of the haptoglobin patterns was accomplished by staining the gels for approximately 10 to 15 min with a solution of *o*-tolidine (1% w/v) dissolved in a mixture of ethanol, glacial acetic acid, and 1% hydrogen peroxide (10:15:10). The electrophoretograms were then photographed (Figs. 1 to 4).

Normally, each molecule of haptoglobin is capable of binding two hemoglobin molecules. Haptoglobin in plasma may be free of hemoglobin, partially saturated, or, in some cases, fully saturated with hemoglobin. The various degrees of saturation are marked by significant differences in the relative molecular weights of the haptoglobin-hemoglobin complexes. However, the electrophoretic separation of the various haptoglobin polymers that compose the different phenotypes depends primarily on differences in the molecular weights of the polymers. Therefore, it is imperative that all haptoglobin molecules be entirely saturated with hemoglobin prior to electrophoresis to avoid the production of additional spurious bands from unsaturated haptoglobin. Thus, in the analysis of haptoglobin phenotypes in blood plasma, it is common practice to add enough hemoglobin in vitro to insure complete saturation of the haptoglobin. Of course, in bloodstains, haptoglobin is already fully saturated with hemoglobin released in copious amounts from erythrocytes.

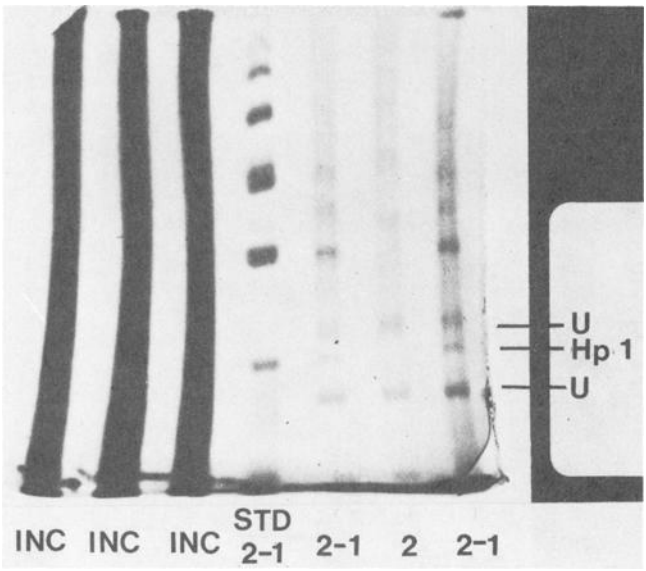

FIG. 1—*Three bloodstains, seven months old, on cotton cloth. The inconclusive* (INC) *results on the left were obtained from unextracted solutions, and the results on the right were obtained from bloodstain solutions extracted with chloroform. The Hp 1 and unprecipitated* (U) *bands are marked.*

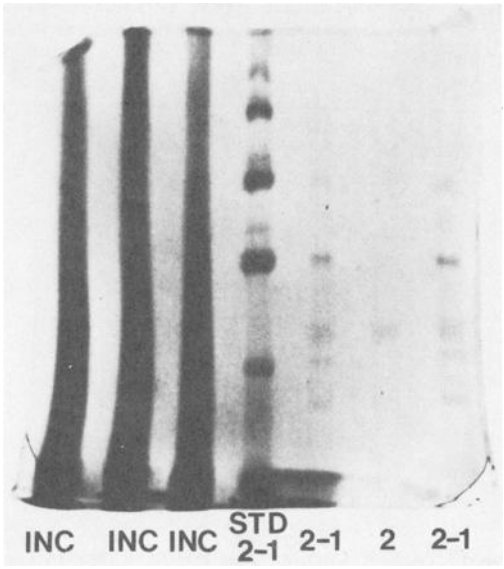

FIG. 2—*Bloodstains, seven months old, from the same three specimens as in Fig. 1, on wool fabric. The inconclusive* (INC) *results on the left were obtained from unextracted solutions, and the results on the right were obtained from bloodstain solutions extracted with chloroform.*

In view of the relative importance of hemoglobin binding to the success of haptoglobin analysis, it was necessary to investigate what effect the chloroform extraction procedure might have on the hemoglobin bound to haptoglobin. Quite obviously, the removal of any bound hemoglobin caused by exposing the haptoglobin to chloroform could potentially

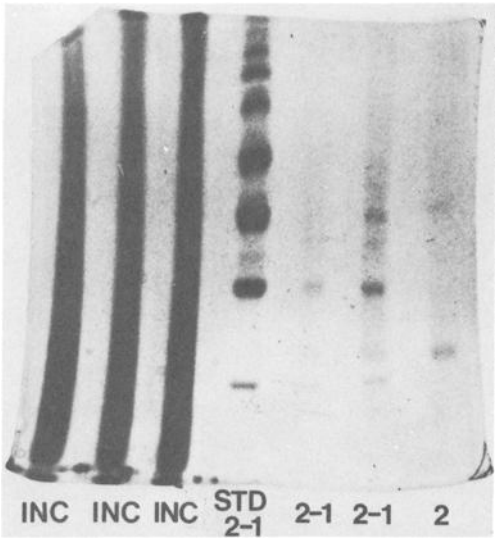

FIG. 3—*Three bloodstains, one year old, on cotton cloth. The inconclusive* (INC) *results on the left were obtained from unextracted solutions, and the results on the right were obtained from bloodstains solutions extracted with chloroform.*

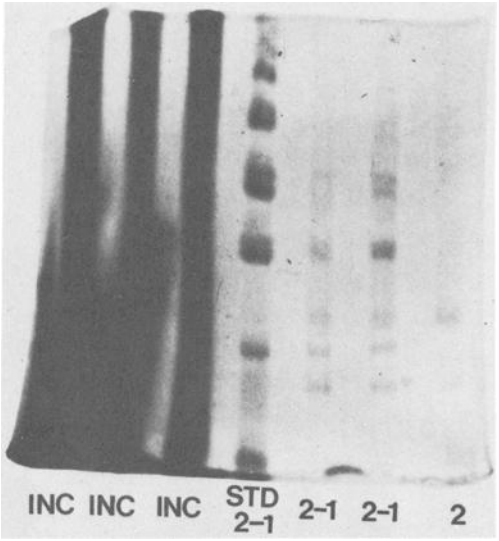

FIG. 4—*Bloodstains, one year old, from the same three specimens as in Fig. 3, on wool fabric. The inconclusive* (INC) *results on the left were obtained from unextracted solutions. and the results on the right were obtained from bloodstain solutions extracted with chloroform.*

affect the electrophoretograms adversely, leading to difficult, if not erroneous, interpretation of the haptoglobin phenotypes. To study the effect of chloroform on fully saturated haptoglobin, the following experiment was conducted.

Fresh plasma specimens from persons of the three common phenotypes, Hp 1, Hp 2, and Hp 2-1, were each mixed with normal hemoglobin, Hb A, sufficient to saturate the hap-

toglobin in the plasma. Each sample was divided into two aliquots and, as with the bloodstain solutions, chloroform extraction was performed on one aliquot but not on the other. Both the extracted and unextracted plasma samples were then analyzed by electrophoresis, stained, and photographed in the same manner as the bloodstain samples (Fig. 5).

Results and Discussion

Bloodstains ranging in age from approximately six weeks to two years, deposited on wool and cotton fabrics, were accurately phenotyped in haptoglobin by electrophoresis after the samples were prepared by a newly adapted chloroform extraction procedure. By contrast, the electrophoretic results from the same bloodstains not subjected to the chloroform extraction method were heavily streaked and, for stains older than six weeks, wholly uninterpretable.

Attempts to phenotype three bloodstains over two years old, with and without chloroform extraction, were unsuccessful.

The chloroform extraction procedure has been shown to eliminate the dark streaking caused by deteriorated hemoglobin present in dried bloodstains. In addition, it should be recognized that the hemoglobin bound by haptoglobin is apparently not affected by this procedure, as evidenced by the electrophoretogram in Fig. 5, which clearly illustrates the unaltered patterns of the phenotypes Hp 1, Hp 2, and Hp 2-1.

It is also important to call attention to the two distinct bands located directly anodic and cathodic of the monomeric haptoglobin band commonly referred to as the Hp 1 band (the single fast-moving band observed with the Hp 1 phenotype) (Fig. 1). These unidentified bands have also been observed in the electrophoretograms of those bloodstains, analyzed without chloroform extraction, where streaking has not totally obscured the bands. In other words, they do not appear to be produced by the chloroform extraction procedure itself. Inasmuch as they demonstrate peroxidase activity, they are presumably forms of hemoglobin or its derivatives. Because these bands can be recognized easily by their characteristic elec-

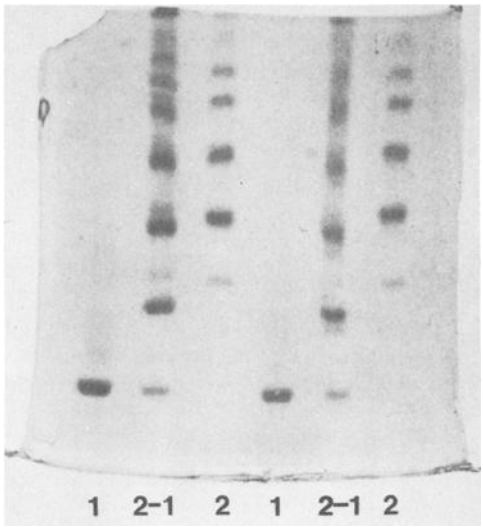

FIG. 5—*Three fresh plasma specimens with Hb A added; the three results in the left were unextracted samples, and the three on the right were extracted with chloroform.*

trophoretic mobility and are well separated from the haptoglobin monomer, they do not interfere with the identification of the haptoglobin phenotypes. Investigation to determine the identity of these bands is continuing.

There are numerous methods of electrophoresis for haptoglobin analysis, some of which call for dissolving bloodstains in a buffered solution rather than distilled water or 10% sucrose solution. It should be remembered that the solubility of proteins is greatly affected by changes in pH, temperature, ionic strength, and dielectric constant of the medium. To preclude the inadvertent precipitation of haptoglobin, the use of a buffered solution in the extraction procedure should be tested adequately before it is substituted in the chloroform extraction method.

Summary

A bloodstain extraction procedure that improves the analysis of haptoglobin in dried bloodstains has been developed. The streaking of electrophoresis gels caused by deteriorated hemoglobin can be eliminated by incorporating chloroform in the bloodstain extraction procedure. The method is easier to execute than previously published techniques for eliminating the adverse effects of deteriorated hemoglobin on the analysis of haptoglobin. Bloodstains up to two years old were correctly phenotyped in haptoglobin by this method.

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