TECHNICAL NOTE

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Detection of Haptoglobin from Concentrated Urine Samples by Enzyme Immunoassay

REFERENCE: Roy, R., **"Detection of Haptoglobin from Concentrated Urine Samples by Enzyme Immunoassay,"** *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 5, Sept. 1991, pp. 1580–1585.

ABSTRACT: The detection of haptoglobin (Hp) from serum and bloodstains is utilized extensively in forensic science laboratories in order to include or exclude possible donors. There is an increasing need to make the same discriminations utilizing genetic markers from urine samples. This paper describes the use of enzyme immunoassay and Western blotting (electrophoretic) techniques to determine Hp phenotypes from concentrated urine samples.

Serum and urine specimens were collected from volunteer donors. The serum sample from each donor was typed for Hp. The urine specimens were concentrated 3000-fold from the starting volume of 15 mL to a final volume of 5 μL and applied to the gradient polyacrylamide gels. This procedure allows the separation of Hp samples into the three common phenotypes as well as the other rare variants found in humans.

The Western blotting electrophoretic technique was used to achieve the transfer of Hp bands from the gels to the nitrocellulose membranes. Enzyme immunoassay with goat anti-Hp antiserum and rabbit anti-goat immunoglobulin alkaline phosphatase conjugate were used to identify the Hp bands from the concentrated samples. Specimens stored for six months at −22°C were also concentrated and typed successfully.

Recent implementation of drug-screening policies has resulted in an increase in the submission of substituted urine specimens. The above procedure can be used to detect an additional genetic marker from urine samples and thus facilitate the identity of the donor.

KEYWORDS: forensic science, genetic typing, haptoglobin, immunoassay, enzyme immunoassay, Western blotting, urine

Liquid urine samples are often submitted to forensic science laboratories for blood group antigen determination. Urine samples are also submitted for toxicological purposes. Due to the recent increase in surreptitiously substituted urine samples in the drug-screening laboratories, it has become necessary to verify the origin of such specimens. The genetic markers identified from such (submitted) specimens can be used to include or exclude possible donors. The vitamin D-binding serum protein group-specific component

Received for publication 10 Sept. 1990; revised manuscript received 5 Feb. 1991; accepted for publication 8 Feb. 1991.

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has been detected in low levels in urine samples. The detection of various phenotypes of group-specific component (Gc) from concentrated urine samples has been possible by using an immuno-blot procedure [*1*].

Haptoglobin (Hp) is an α^2 serum glycoprotein. In humans, Hp, a hemoglobin-binding protein, manifests three main phenotypes: Hp 1, Hp 2-1, and Hp 2 [*2*]. It has been shown to remain stable for a prolonged period of time [*3*]. Hp has a high "discriminatory power" of 0.60 [*4,5*].

Three alternate procedures were previously reported for concentration of urine samples for determination of blood group substances: Sephadex® G-200-50,[2] Lyphogel® polyacrylamide gels,[3] and Centriprep-30®[4] [*6*]. In the present study, two of these procedures—Lyphogel gel beads and Centriprep-30—were used to concentrate urine samples up to 3000-fold. The presence of Hp bands in the concentrated samples was detected using Western blotting and immunoblot assay after electrophoresis.

Materials and Methods

Serum was collected from liquid blood samples from volunteer donors, and Hp phenotypes were determined by methods described previously [*7*]. For staining of Hp bands, *o*-dianisidine and *o*-tolidine dyes were used as described previously [*7,8*].

Urine samples were collected in sterile specimen cups from each volunteer donor from either one or two miturations for a total volume of 30 mL. These samples were divided into two groups of 15-mL specimens and kept in separate vials. Each of these 15-mL specimens in both groups was then concentrated individually by one of the following methods.

Lyphogel (Gelman No. 51030) Polyacrylamide Gel Beads

Lyphogel gel beads were used as the first method of concentration. These beads were dropped directly into the 15-mL urine sample in a beaker. One gram of the beads absorbs up to 5 g of water from the aqueous urine. To avoid fluid evaporation, with concomitant protein deposition on the sides of the container, the beaker was covered tightly with parafilm during concentration.

When the sample volume appeared to be approximately 500 μL, it was centrifuged at 5000 rpm for 5 min. A volume of 500 μL of the supernatant aqueous layer was pipetted out in a centrifuge tube. This volume was further concentrated in a Savant SpeedVac apparatus until the remaining volume was approximately 5 μL.

Amicon Microconcentrator Centriprep-30

Fifteen-millilitre urine samples were each reduced to approximately 500 μL after the third spin in Centriprep-30. For this study, a swinging bucket rotor was used, and the liquid urine specimens were concentrated until the final volume of each concentrate was approximately 500 μL. The specimens were then further concentrated to 5 μL using the SpeedVac apparatus, as described in the previous procedure.

Electrophoresis, Western blotting, and Detection

The tank buffer for electrophoresis was prepared by dissolving 21.8 g/L of glycine (0.29*M*) and 4.5 g/L (0.037*M*) of Tris base [tris(hydroxymethyl)aminomethane]. The

[2]Manufactured by Sigma, St. Louis, MO.
[3]Manufactured by Gelman, Ann Arbor, MI.
[4]Manufactured by Amicon Corporation, Beverly, MA.

resultant pH of the buffer was 8.3. Commercially prepared 2 to 16% polyacrylamide gradient gels from Pharmacia-LKB (PAA 2/16) were run as horizontal slab gels on a SERI electrophoresis apparatus. The gel sizes were 75 by 75 mm, with a thickness of 2.7 mm.

The following solutions and buffers were used for the transfer of Hp onto nitrocellulose membranes (Western blotting) and for enzyme immunoassay: Trans-blot tank buffer and gel equilibration buffer (Tris-glycine buffer) were prepared by dissolving 7.27 g/3 L Tris base (20m*M*) and 33.78 g/3 L glycine (150m*M*) in 600 mL of methanol and deionized water. The resultant pH of the buffer was 8.4. Tris buffered saline (TBS) was prepared by dissolving 1.21 g/1 L Tris base (10m*M*) and 29.22 g/1 L sodium chloride (NaCl) in deionized water. The pH of the buffer was adjusted to 8.0 with hydrochloric acid (HCl). Tween Tris buffered saline (TTBS) (0.1% Tween-TBS) was prepared by adding 1 mL of Tween 20 (Bio-Rad) to 1 L of TBS.

Blocking solution (1% gelatin-TBS or 0.5% BSA-TBS) was prepared by dissolving 1 g of gelatin in 100 mL of TBS. (Gelatin requires slight heat for dissolving.) Also, 0.5 g of BSA in 100 mL of TBS can be used (Bio-Rad and Ortho-Diagnostics). The antibody buffer was 1% gelatin-TBS or 0.5% BSA-TBS. First, antibody was used as 1:100 dilution of anti-human Hp (goat) in antibody buffer (ATAB). A second antibody was used as a 1:2500 dilution of alkaline phosphatase rabbit anti-goat (ATAB).

For color development, the following buffer and reagents were used. Alkaline phosphatase buffer was prepared by dissolving 0.84 g of sodium bicarbonate ($NaHCO_3$) and 0.0203 g of magnesium chloride ($MgCl_2.6H_2O$) in a total volume of 100 mL with deionized water. The pH was adjusted to 9.8 with sodium hydroxide (NaOH). An alternate buffer can be used by mixing 10 mL solution of 1*M* Tris-HCl (pH 9.5), 2 mL solution of 5*M* sodium chloride (NaCl), and 500 μL solution of 1*M* $MgCl_2$ in a total volume of 100 mL with deionized water.

NBT stock solution was prepared by dissolving 30 mg of *p*-nitro blue tetrazolium chloride in 1 mL of 70% *N*,*N*-dimethylformamide (DMF) (Sigma Chemical). BCIP stock solution was prepared by dissolving 15 mg of 5-bromo-4-chloro-3-indolyl phosphate (disodium salt) in 100% DMF (Sigma Chemical).

Color developing solution was prepared immediately prior to its use by mixing 20 mL of alkaline phosphatase buffer, 132 μL of NBT stock solution, and 68 μL of BCIP stock solution. The color development was stopped by rinsing in deionized water. Alternatively, a solution of 20m*M* Tris-HCl (pH 8.0) and 5m*M* ethylenediaminetetraacetic acid (EDTA) can also be used as a reaction stop buffer.

A volume of 5 μL of the concentrated urine sample was applied onto No. 3 Whatman filter paper strips (5 by 1 mm) and gently placed on top of the gel surface approximately 1 cm from the 2% cathodic edge. Two sponges, one on each end of the gel, were used as wicks to provide contact with the buffer in the tanks. Serum samples were diluted 1:150, and 3 μL was used on filter strips, as described for urine sample application.

Following sample application, electrophoresis was performed at 4°C using a Pharmacia-LKB electrophoresis power supply at 125 V for 17 h or until the hemoglobin bands were at the 16% anodic end of the gel.

Following electrophoresis, Western blotting was performed. The transfer and incubations, described below, were carried out at room temperature. The following steps were followed for Western blotting and enzyme immunoassay:

The gel was equilibrated in Tris-glycine buffer for 60 min with one change after 30 min while being gently shaken on a rotator. It was then transferred onto the gel holder of a Bio-Rad Trans-Blot apparatus. A piece of nitrocellulose membrane (Bio-Rad 0.45 μm) previously soaked in the Tris-glycine buffer was laid on top of the gel, while care was taken to remove any air bubbles trapped between it and the membrane. Western blotting (electrophoretic) transfer of the Hp molecules was achieved by overnight (16 h) transfer in a Bio-Rad Trans-Blot apparatus containing Tris-glycine-methanol buffer at 25 V with a Bio-Rad power supply.

After 16 h, the membrane was removed and the free sites were blocked by incubating in 0.5% BSA-TBS or 1% gelatin-TBS for 1 h. The nitrocellulose membrane (blot) was then incubated for 2 h with the first antibody in 0.5% BSA-TBS or 1% gelatin-TBS. The blot was washed in four changes of TTBS for 15 min each and incubated for 2 h with the second antibody in 0.5% BSA-TBS or 1% gelatin-TBS. The blot was washed in four changes of TTBS for 15 min each. The peroxidase activity was visualized by submerging the blot in the alkaline phosphatase color developing solution and rinsing it in distilled water to stop the reaction. The blots can be left in the color developing solution for a few minutes to overnight depending on the desired intensity of the Hp bands. Band intensity increases with the increase of time in the developing solution. The nitrocellulose blots can be air-dried and stored in the dark at room temperature.

Results and Discussion

Serum and urine samples were collected from individual donors. The serum samples were typed first for their Hp phenotype determination. Each individual's corresponding urine sample was then concentrated by each of the two procedures described above. The results of Hp phenotype determination from urine samples concentrated by both procedures corresponded with the Hp phenotypes from the serum (Fig. 1). Both Lyphogel and Centriprep-30 concentration procedures were equally successful in their ability to concentrate various urine samples. Hp phenotypes were determined correctly using both procedures.

Urine samples stored at −22°C for six months were typed successfully after concentration with either Lyphogel or Centriprep-30. Hp phenotypes were correctly determined from samples stored at room temperature for four weeks. Samples stored for more than four weeks showed weak bands or no band patterns (Fig. 2).

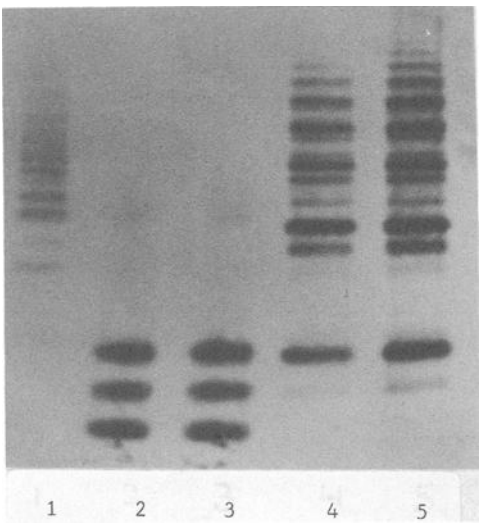

FIG. 1—*Hp phenotypes on nitrocellulose membrane from urine samples concentrated immediately after collection. The phenotypes from left to right are* (1) *Hp 2 from urine concentrated by Lyphogel,* (2) *Hp 1 from serum diluted 1:150,* (3) *Hp 1 from urine concentrated by Centriprep-30,* (4) *Hp 2-1 from urine concentrated by Lyphogel, and* (5) *Hp 2-1 from fresh serum diluted 1:150. The cathode is on top. The direction of migration is from cathode to anode.*

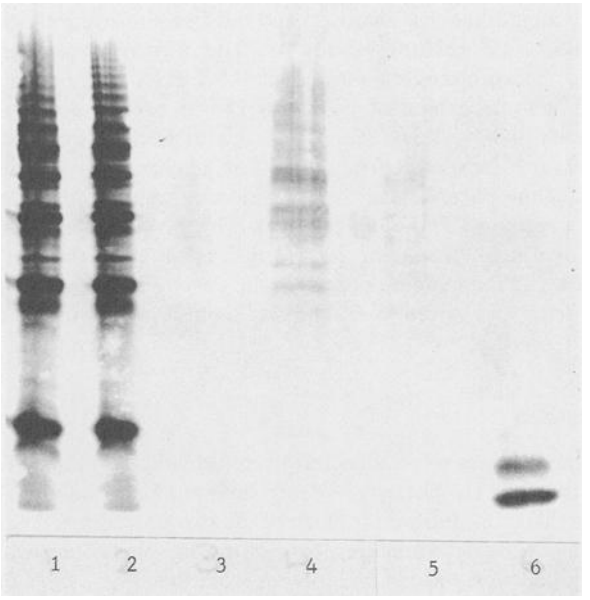

FIG. 2—*Hp phenotypes on nitrocellulose membrane from urine samples stored at room temperature and concentrated by Lyphogel. The phenotypes from left to right are* (1) *Hp 2-1 from serum diluted 1:150,* (2) *Hp 2-1 from urine stored for four weeks,* (3) *Hp 2-1 from urine stored for eight weeks,* (4) *Hp 2-1 from urine stored for five weeks,* (5) *Hp 2 from urine stored for six weeks, and* (6) *Hp 1 from urine stored for four weeks. The cathode is on top. The direction of migration is from cathode to anode.*

Lyphogel absorbs water and salts from aqueous solutions and excludes proteins and other substances with molecular weights of 20 000 or more from its inner volume. By using absorbent polyacrylamide hydrogel beads, the time-consuming step of dialysis was avoided. The concentration of 3000-fold by using both Lyphogel and Speed-Vac rendered the Hp typing possible. The use of Centriprep-30 also avoided the necessity for dialysis and removal of salts. Concentration and desalting occurs at the same time and samples are reduced to 500 μL. Further concentration of these to 5 μL for electrophoresis was necessary for Hp visualization.

Interpretation of the phenotypes from aging urine samples should be made with extreme caution. In general, older urine specimens showed some streaking. Special care is essential when concentrating urine specimens which have been left at room temperature so that no precipitate remains in the supernatant. The 1 band of the Hp 2-1 type appeared to be less stable than the other bands. The 1 band of Hp 1 and Hp 2-1 types appeared to be more likely to disappear from aged urine specimens when stored at room temperature. A type Hp 1 might thus exhibit no activity. A type Hp 2-1 can be mistakenly interpreted as an Hp 2.

A few of the urine samples concentrated immediately upon collection by both of the procedures showed no Hp activity. No explanation can be offered at this time for either the complete loss or the loss of some of the bands from the Hp proteins in those urine samples in this study. Bacterial conversion of urea to ammonia has been reported to occur in urine specimens stored at room temperature [*9*]. Whether this or some other phenomenon is responsible for the loss of the bands is unclear at this time. The changes that occur in urine after its excretion will be investigated at a later date. In some samples, the Hp phenotype from the same donor varied slightly in pattern when urine specimens were collected on different days. It is possible that some of the Hp proteins excreted in

the urine specimens were complexed with hemoglobin. Experiments were carried out in which hemoglobin was added to the concentrated samples prior to their application on the gel. These specimens yielded a slightly different banding pattern than those applied directly and without added hemoglobin.

The antigenic activity of the urine Hp in the 5-μL final concentrates corresponded to that of a 3000-fold concentration of the original samples and was nearly equal to the 150-fold diluted serum samples used in this study.

Conventional detection procedures using *o*-tolidine and *o*-dianisidine failed to detect any bands from the concentrated samples. The sensitivity of the enzyme immunoassay was essential in the visualization of the Hp bands. The alkaline phosphatase immunoassay has been known to detect the Hp bands in serum diluted 1:256 [*10*]. It is utilized in this study for detection of Hp bands in urine samples with successful results. The method described herein can be used with success in forensic cases in which samples are submitted for serological or toxicological purposes. Detection of Hp phenotypes from urine provides the forensic serologist with additional information about a genetic marker obtained by a sensitive test from a body fluid from which, until recently, only blood group substances could be determined.

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