TECHNICAL NOTE

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Development of a Radial Gel Diffusion Technique for the Identification of Urea in Urine Stains

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ABSTRACT: A radial gel diffusion method utilizing urease and bromthymol blue has been developed for urine stain identification. Urea, present in urine in relatively high concentrations, can be detected from urine stain extracts. This technique provides both qualitative and quantitative results, and is sensitive enough to detect 0.078 $\mu g/\mu L$ of urea.

KEYWORDS: criminalistics, urine, identification systems, urine stain identification, urea, urease, bromthymol blue, radial gel diffusion

Urine is composed of many substances including inorganic ions, proteins, uric acid, creatinine, and urea. Urea is the major end product of protein metabolism and represents 80 to 90% of the total urinary nitrogen [*1*]. Since urea is one of the major components of urine, ranging from 10 to 30 mg/mL [*2*], emphasis was placed on developing procedures to detect urea in urine using urease, as a means to identify urine stains.

Urease is a stable enzyme extracted from beans, which specifically acts upon urea. In the presence of urease, urea is hydrolyzed to carbonic acid and ammonia [*1*]:

$$\begin{array}{l} NH_2 \\ | \\ C = 0 + 2H_2O \\ | \\ NH_2 \end{array} \xrightarrow{\text{Urease}} \begin{array}{l} OH \\ | \\ C = 0 + 2NH_3 \\ | \\ OH \end{array}$$

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This reaction is commonly monitored by detecting the basic pH change as ammonia is liberated. Bromthymol blue is used as the acid-base indicator. There have been several methods developed that detect urea. One suggested method involves the evaporation and crystallinzation of urea followed by the addition of nitric acid to form characteristic urea nitrate crystals [3]. In the xanthydrol test, dianthylurea crystals are formed with the addition of glacial acetic acid and xanthydrol, and can be observed microscopically [4]. Chloroplatinic acid is also used in urea determinations. A water extract of stained material is reacted with urease. Chloroplatinic acid is then added and with the evolution of ammonia, characteristic octahedral crystals of ammonium platinic chloride are formed [5]. Ishler et al [6] used ammonia detection by urease with manganese dioxide and elimentary silver. A procedure developed by Rhodes and Thornton [7] utilizes a 0.005% solution of paradimethylaminocinnamaldehyde, and in the presence of urea, a dark pink or red color is observed. Taylor and Hunt [8] have developed a procedure for identifying human urine that uses a specific rabbit anti-human urine antibody (Tamm-Horsfall protein) in a solid phase radioimmunoassay technique. Bromthymol blue, a popular acid base indicator is utilized in a paper test with urease, as a qualitative test for urea [9]. Bromthymol blue has also been incorporated into a similar procedure using an agar gel to detect urea on wheat grains [10].

In most crime laboratories, dried urine stains are tentatively identified by determining the presence of urea. The most common technique in practice is the "urea nitrogen" test (Berthelot reaction), which employs the use of a commercial kit of prepared chemical reagents [11]. The technique generates ammonia from urea and a positive reaction results in the production of the typical deep blue color of indophenol. The reaction is carried out in test tubes and requires rather large amounts of stained material by forensic science standards. That is to say, the test is not particularly sensitive for the urine stains normally encountered as evidence in criminal cases. Most of the procedures available to date for urine identification are time-consuming, tedious, costly, and impractical for the purpose of forensic science laboratories. In this present study, we have developed a procedure for the identification of urine stains that requires very small samples, is less time-consuming, and requires few chemicals. The method is sensitive enough to detect 0.078 $\mu g/\mu L$ of urea.

Materials and Methods

The following reagents are needed. All of the chemicals used were analytical grade.

Bromthymol Blue (BTB)

Weigh 150 mg of BTB (Sigma #B8630) and add 10 mL of distilled water. Add one drop of phosphoric acid (1:10) and dissolve. pH to 5.8 to 6.0 with 0.1*N* sodium hydroxide (yellow-green).

Urease

Weigh 25 mg of urease (Sigma #U-4002, 7000 units/g) and dissolve into 10 mL of distilled water (0.2 units/plate).

Agar

Put 2% agar (w/v) in distilled water. If necessary, adjust pH of distilled water to 5.8 to 6.0 before adding agar. Boil gently and cool to 48 to 50°C.

Test Plates

Add 1.0 mL of BTB solution and 5.0 mL of urease solution to agar (100 mL) and mix. Adjust the pH to 5.8 to 6.0 with 0.1*N* sulfuric acid. Pour approximately 3.0 mL of the liquid agar

into plastic petri dishes (50 by 9 mm) and let solidify. (Makes approximately 25 plates.) For the preparation of the blank plates prepare as above omitting the urease. After solidification, a pasteur pipette connected to a water suction apparatus is used to make wells (1.0 mm in diameter) in the agar. The covered plates are stored upside down in the refrigerator at 4°C.

Random urine samples of 25 individuals were collected on filter paper and dried. For qualitation purposes, the stains were cut to approximately 1 cm^2 in size and extracted in 0.2 mL of distilled water at room temperature for 15 min. The extracts (2 μL) were pipetted into the sample wells of the test plates and blank plates and allowed to diffuse for 10 min. At 10 min the ring diameters were measured. For the standard curve, urea standards of varying concentrations 0.039 to 2.5 $\mu g/\mu L$) were used. One microlitre of each solution was pipetted into the agar well and allowed to diffuse. After 10 min the diameters of the rings were measured. Using the standard curve, urea can be quantitated. The 25 urine stains were cut to 0.5 cm^2 in size and extracted in 0.2 mL of distilled water at 25°C for 15 min for quantitation purposes. One microlitre of the extract was pipetted into the test and blank plates and allowed to diffuse for 10 min after which the ring diameters were measured. Other body fluids also contain urea. Several body fluid stains were tested for reactivity using the qualitative procedure previously mentioned. The body fluid extracts that were tested include saliva, vaginal extracts, tears, serum, semen, and perspiration.

Results

Of the 25 random stains qualitatively tested, dark blue radial patterns were observed in the test plates (see Fig. 1). The ring diameters ranged in size from 5.0 to 7.0 mm. All 25 of the blanks showed no color reaction. Since this method uses the urea specific enzyme urease, all samples were determined to be positive for urea, as the blanks were negative.

A standard curve was prepared using urea standards of varying concentrations. The diameter of the reaction circle is proportional to the square root of the concentration of the diffusing reactant. Therefore concentration of urea in micrograms/microlitres versus the ring diameter squared in square millimetres was plotted on a graph paper; the curve was more hyperbolic than linear. Then the log of the urea concentraiton ($\mu g/\mu L$) versus the ring diameter (mm) was plotted on standard graph paper, which was linear. The linearity range of urea determined by

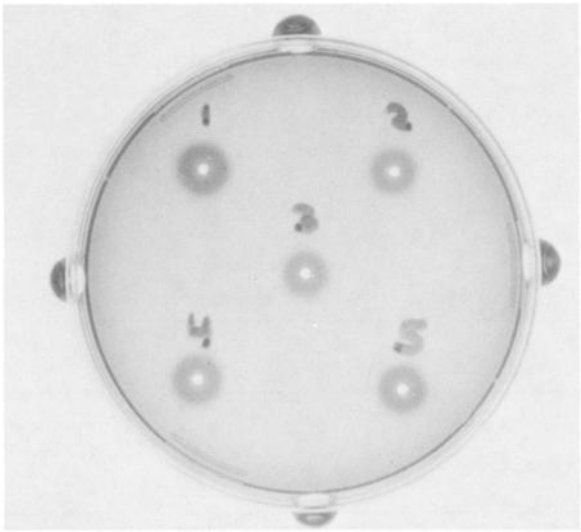

FIG. 1—*Urine stains of 1 cm^2 extracted in 0.2 mL of water.*

this standard curve is 0.078 to 0.62 $\mu g/\mu L$ of urea (see Fig. 2). Because of the small concentrations of urea, the radial patterns that developed were very light blue in color making measurements somewhat difficult.

Of the 25 urine extracts used for quantitation, 24 samples did fall within the linear range when tested, ranging from 0.14 to 0.625 μg/well of urea. One of the samples fell below the established linearity range. The extracts were incubated at 25 and 37°C. No significant difference was observed in the ring diameters.

When qualitatively tested, the saliva and vaginal extracts were negative, however, tears, semen, serum, and perspiration stains developed light blue radial patterns as a result of their urea contents. (Bloodstains were eliminated because of visible color of the bloodstain extract.) The reactive body fluids were retested using stains 0.5 cm^2 in size, and were extracted in 0.4 mL of distilled water. The reactivity of tears and serum was eliminated. Semen had visible rings measuring 4.0 mm in the test and blank plates, while perspiration had a visible ring pattern in the test plate only, measuring 4.0 mm (see Table 1). The recovery of urea from stains was also determined. Of a prepared urea standard (0.33 $\mu g/\mu L$) 120 μL was pipetted onto a piece of filter paper and allowed to dry. The filter paper was then extracted into 300 μL of distilled water for 15 min at room temperature. One microlitre was then pipetted into the well of the test plate and allowed to diffuse for 10 min, at which time the ring diameter was measured to be 4.3 mm. The recovery of urea from the stain was determined by our standard curve to be approximately 85%.

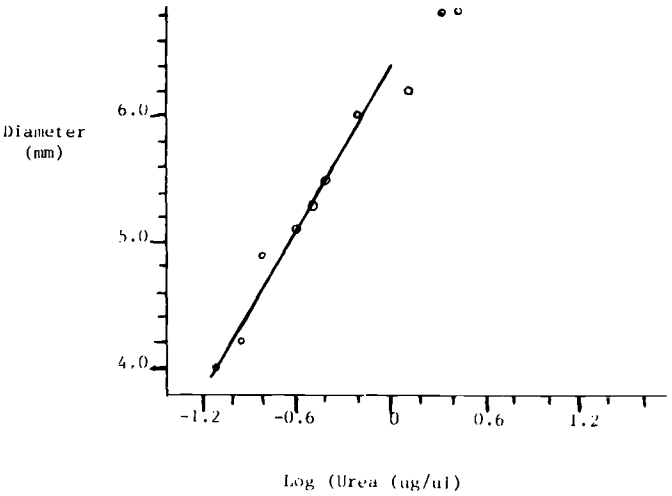

FIG. 2—*Standard curve for urea.*

TABLE 1—*Qualitative testing of tears, semen, serum, and perspiration stains.*

Body Fluid Stain 1/2 cm^2 in 0.4 mL of Distilled Water	Test Ring Diameter, mm	Blank Ring Diameter, mm
Tears	negative	negative
Semen	4.0	4.0
Serum	barely visible[a]	barely visible[a]
Perspiration	4.0	negative

[a]The radial pattern was too light in color to be measured.

Discussion

All urine stains tested developed blue radial patterns in the gel and were determined to be positive for urea as the blanks were negative. However, a positive color reaction in the blank would invalidate this test. The standard curve was determined to be linear in the range of 0.078 to 0.62 $\mu g/\mu L$ of urea, when plotted as log of concentration [c] versus diameter, and could be used as a calibration curve also. All random urine stains, when cut to 0.5 cm^2 in size and extracted in 0.2 mL of distilled water, yielded concentrations that fell within the linearity range established by the standard curve, enabling quantitation. Smaller stains may be extracted in less distilled water to obtain a color reaction.

Other body fluids were tested for reactivity. Tears, semen, serum, and perspiration stains did develop light blue radial patterns because of their urea contents. However, when retested, the reactivity of tears and serum was eliminated. Semen and perspiration stains still show a reaction with the semen stain also developing a light blue radial pattern in the blank plate. This could possibly be due to the pH of the semen, since any extract with an alkaline pH would develop a blue color reaction in the presence of the acid-base indicator bromthymol blue.

Also note that the urea recovery from the stain was determined to be approximately 85%. Centrifugation of the sample after the 15-min extraction time may (piggyback) increase the recovery rate.

The average amount of urea present in the urine stains tested was calculated to determine a minimum value of urea in micrograms/microlitres, which could be used as a minimum threshold value for the identification of urine stains. The average amount of urea present in each stain was determined to be 0.30 $\mu g/\mu L$. The values ranged from 0.14 to 0.625 $\mu g/\mu L$ of urea. Therefore, it is highly recommended that a known urea standard containing 0.14 $\mu g/\mu L$ of urea be run as a threshold standard. If a blue color reaction develops below this level, it should be disregarded as a urine stain. It is also recommended that the following list of controls be run each time when an unknown stain is tested:

(1) unknown stain extract;
(2) unstained control;
(3) saline, as a blank; and
(4) positive control, (0.14 $\mu g/\mu L$ of urea).

Plates can be made and stored three months at 4°C in the refrigerator upside down ready to use whenever required. Optimum time for ring development is 10 min, after which diffusion becomes excessive and the color starts to diminish. Therefore, it is recommended that the results should be recorded at 10 min.

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