New Dip-and-Read Test for Determining Leukocytes in Urine


A new reagent strip for the determination of leukocytes in urine (LEUKOSTIX®-Ames) is described. The test is based on the esterase activity in leukocytes as a marker. Upon contact between the reagent matrix and a urine containing leukocytes, an amino acid ester is hydrolyzed by the esterase to its corresponding alcohol. The free alcohol then couples with a diazonium salt to produce a purple azo dye. The relative concentration of leukocytes in the urine is obtained by visually comparing the strip reaction with a color chart. Performance of the strip was evaluated in a clinical study involving eight different sites and 867 urine specimens. The comparison method was sediment microscopy; specimens containing five cells or more per high-power field were considered to be positive. Sensitivity was 76.3%, specificity 90.8%. Performance was comparable with that of the CHEM-STRIP® LN (Boehringer-Mannheim Diagnostics, Inc.) leukocyte test, which we evaluated concurrently.

For many reasons, routine urinary microscopic examination is being questioned as a first-line method of screening for urinary tract disease (1-7). Considerations of economics, efficiency, staffing, and new government regulations have caused more and more clinical laboratories to seek alternative methods. This is particularly true in the case of leukocyte determinations, and to this end an appropriate new dry-reagent test is reported here.

The test exploits the activity of a naturally occurring esterase in leukocytes, which acts on an amino acid ester incorporated into the strip matrix. This compound, 3-hydroxy-5-phenylpyrrole, N-tosyl-l-alanine ester, is hydrolyzed forming the corresponding 3-hydroxy-5-phenylpyrrole. This alcohol in turn couples with a diazonium salt, 1-diazo-2-naphthol-4-sulfonic acid, to form a purple azo dye product (Figure 1). The concentration of leukocytes in the urine is then estimated by comparing the color formed on the strip with the color chart provided. We believe such a test should be useful for confirming microscopic examinations with respect to the presence or absence of leukocytes in urine. The strip test does not rely on the absolute presence of intact leukocytes to function; thus, cell lysis in the sample will not compromise the test result.

We describe here the chemical and performance characteristics of this test, along with some results of interference studies. We also report the findings of eight different clinical laboratories regarding the sensitivity and specificity of this test relative to two currently used methods: the urinary sediment microscopic count and the Boehringer-Mannheim leukocyte test strip. A preliminary report has appeared (8).

Materials and Methods

Reagents

1-Diazo-2-naphthol-4-sulfonic acid (Sigma Chemical Co., St. Louis, MO) was recrystallized before use. 3-Hydroxy-5-phenylpyrrole, N-tosyl-l-alanine ester, is manufactured in-house. All other materials were ACS-reagent grade or the best available.

Procedures

Reagent paper for the manufacture of LEUKOSTIX® reagent strips is impregnated in a two-step process. In the first, the filter paper is passed through a solution containing a buffer and various stabilizers and enhancers. The treated paper is then dried and passed through a second solution containing 1-diazo-2-naphthol-4-sulfonic acid and 3-hydroxy-5-phenylpyrrole, N-tosyl-l-alanine ester, in organic solvent. The dried paper is then cut into 5.1-mm squares and mounted on 5.1 x 82.5 mm plastic strips by means of double-faced adhesive.

Polymorphonuclear leukocytes were isolated from pooled specimens of fresh blood from healthy male donors (9).

Reagent-strip analyses. Strips were analyzed instrumentally with a Macbeth 1500 reflectance colorimeter (Macbeth Division, Kollmorgen Corp., Newburgh, NY) or a custom-built rapid-scanning reflectance spectrometer (10). These instruments can scan reagent strips from 400 to 700 nm in 20-nm increments extremely quickly. The instruments then convert the spectral data into linearized three-dimensional "color space" points with dimensions of L*, a*, and b* (11), where one unit represents the smallest color change perceivable by a person with normal vision. ∆E, the linear distance in color space between any two points, is taken as the square root of the sum of the squares of the differences between two L*a*b* values. ∆E values between the positive readings and a negative reference reading were determined 2 min after the strips had been dipped into their respective samples. Strips read visually were compared with a standard LEUKOSTIX reagent-strip color chart, also after a 2-min reaction time.

Clinical studies. At each of eight clinical sites, the same lot of LEUKOSTIX reagent strips was used to analyze fresh clinical urine specimens, voided less than 2 h before, for the presence of leukocytes.2 One lot of CHEM-STRIP LN (Boe-

---

2 Details of results of this study are obtainable on request to the authors.
ringer-Mannheim Diagnostics, Indianapolis, IN, with an indoxylcarboxylic acid ester substrate) was also used to analyze the same specimens. Workers at these sites were instructed to screen urines so as to include a higher than routinely encountered percentage of specimens that were positive microscopically. Each of two readers assayed each specimen with each of the two brands of strips. The readers were instructed to compare the leukocyte reagent pad with the color chart 2 min after dipping the strip in the urine and to record the result. Each urine was also evaluated by the high-power field (sediment microscopy) procedure as the comparison method. Because this procedure was to be performed in the manner usually followed at each site, it varied from site-to-site. Routine microscopic and chemical urinalysis data were also collected for each specimen, the latter by use of N-MULTISTIX® SG reagent strips (Ames).

Interference studies. We assessed possible urinary interferences, by assaying urines to which ascorbic acid, human serum albumin, glucose, bilirubin, indoxyl sulfate, oxalic acid, hemoglobin, or intact erythrocytes were added, and to which isolated granulocytes had already been introduced. We looked for any changes in strip response. We used N-MULTISTIX SG reagent strips to determine relative density.

Results and Discussion

Strip Characteristics

Figure 2 illustrates the spectral characteristics of LEUKOSTIX strip response as a function of reflectance. This display reflects strong, broad-spectrum color responses, peaking in the purple region (as visually perceived) at 560 nm. Broad spectral displays that extend into the higher wavelengths are desirable, because color differences in this area are more readily distinguished by eye and ordinarily are not masked by urine color. The time-course for color development is presented in Figure 3. We use the Kubelka-Munk ratio \( R \), \( K/S = (1 - R)^2/2R \) (where \( K \) is the absorption coefficient, \( S \) is the scattering coefficient, and \( R \) is reflectance), for such kinetic plots, because this allows linearization of the reflectance parameter. Here, except for the highest leukocyte value tested, rates of color development were relatively constant for various cell concentrations during the course of the 2-min reaction. Thus we selected 2 min as the visual reading time for the test, an interval that would allow maximum strip response but still be in line with the fast reading times associated with other urinary test-strip determinations.

Clinical Correlations

With the microscopic count as the comparison method, 867 urines were evaluated with LEUKOSTIX reagent strips. The criteria for a positive microscopic result was five granulocytes or more per high-power field (HPF). Thus, of 393 urines positive microscopically, 300 were positive by strip, giving a sensitivity of 76.3%. Of 474 urines judged negative by microscopy, 384 were negative by strip, giving a specificity of 80.8%. Sensitivity and specificity values for CHEMSTRIP LN by the above criteria were 75.4% and 82.9%, respectively. The above results fall within the range of those previously published (1, 3, 13-16). Figure 4 shows a correlation plot comparing the results with the two strips.

Interference Studies

Interference characteristics were first tested in simulated trials. We were initially concerned with ascorbic acid, which has a strong reducing effect on many indicator reactions. The results indicate no adverse effect on the strip response by ascorbic acid concentrations up to 2 g/L. Although there appears to be a slight accelerative effect of ascorbate at 0.5 to 2.0 g/L in leukocyte-positive urines, test strip results remained negative for urines to which leukocytes were not added.

A slight inhibitory effect by albumin was observed. An albumin concentration of 10 g/L resulted in the loss of up to one-half of a color block response. However, clinical specimens that contain such concentrations of albumin are rare.
In the current study only six were encountered in 866 samples (0.7%). We now believe that this inhibition may be caused by a contaminant common to serum albumin preparations, because later studies with purer preparations of human serum albumin showed less inhibition of the strip test.

Glucose, at 30 g/L, had significant effects on reactivity (Table 1), inhibiting the trace reaction (10 cells per microliter) by 64%. Carbohydrates such as glucose complex with borate (17), the buffering entity in our system, to release a proton. If such complexing and proton release is substantial, the buffering capacity of the strip could be exceeded and make the reaction pH unacceptably low. In this clinical study, the frequency of urines containing 2.0 g/L glucose or greater was 18 in 865 (2.1%), a frequency that, combined with the extent of inhibition involved, we consider clinically important—and we have so stated in our package insert. A similar but less-pronounced effect was noted for CHEMSTRIP LN reaction strips.

We observed a slight inhibition from oxalic acid at 500 μg/mL, and from indoxyl sulfate at 250 to 750 mg/L. There was no interference from bilirubin at 8 or 16 mg/L, erythrocytes at 5000 or 10 000 per microliter, or free hemoglobin at 4.05 or 40.5 mg/L.

Relative density effects. Sensitivity and specificity values were sorted as a function of relative density (specific gravity). The results (Table 2) indicate a bias towards higher sensitivity and lower sensitivity as relative density increases. Decreased sensitivity at low relative density may be a function of lysed leukocytes, thus indicating only an apparent false-positive situation. This phenomenon has been recorded for urines of low osmolality (18) and we have also noted it occasionally in our own laboratory. Loss of sensitivity with increasing relative density could be a result of a general inhibition or possibly a failure of esterase to leak from the cell. Thus the cell counts in the comparison method may be low under these conditions, resulting in skewed sensitivity and specificity. Results for CHEMSTRIP LN (Table 2) show the same bias, to a lesser extent.

Antibiotic effects. During the course of a urinary tract infection, specific antibiotics may be administered, many of which are eliminated unchanged in the urine. We performed a study to assess the effects of such antibiotics on the strip response. The results (Table 3) indicate that five of 17 commonly prescribed antibiotics interfere with the test in some way. Although patients in a screening regime probably would not yet be undergoing treatment, investigators should be aware of these interferences. We have not investigated possible effects of any metabolites of these drugs.

Other Effects

There were two other important findings from our field studies:

Leukocytes disintegrate appreciably as urine specimens become more than 4 h old. Thus cell counts are no longer accurate, and artificially low specificity values are obtained. In such instances, the strips are probably more reliable than microscopy for indicating the presence of leukocytes, because their performance does not depend on cells being intact.

The use of daylight rather than fluorescent light when matching strips to color charts may confuse the reader, resulting in both low specificity and sensitivity.

In a recent independent investigation, taking advantage of these lessons learned in our studies, Scher obtained sensitivity and specificity results of 89.4 and 90.4%, respectively (19).

We thank P. Burmeister for skillful secretarial assistance, C. Davis for expert statistical analysis, and E. Schnabel and J. Travis for many valuable discussions.

References

### Table 1. Interference of Glucose with LEUKOSTIX Reagent-Strip Performance

<table>
<thead>
<tr>
<th>Glucose, g/L</th>
<th>Cells/μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>94</td>
</tr>
<tr>
<td>25</td>
<td>97</td>
</tr>
<tr>
<td>75</td>
<td>91</td>
</tr>
<tr>
<td>200</td>
<td>99</td>
</tr>
</tbody>
</table>

*Results are the percentage of the ΔE values for control samples containing no glucose.

### Table 2. Performance of LEUKOSTIX and CHEMSTRIP LN Reagent Strips as a Function of Relative Density

<table>
<thead>
<tr>
<th>Relative density</th>
<th>No. of urines</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>LEUKOSTIX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.000–1.010</td>
<td>247</td>
<td>88.0</td>
<td>65.5</td>
</tr>
<tr>
<td>1.015–1.020</td>
<td>374</td>
<td>73.7</td>
<td>86.5</td>
</tr>
<tr>
<td>1.025–1.030</td>
<td>243</td>
<td>67.9</td>
<td>87.8</td>
</tr>
<tr>
<td>CHEMSTRIP LN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.000–1.010</td>
<td>247</td>
<td>84.0</td>
<td>77.1</td>
</tr>
<tr>
<td>1.015–1.020</td>
<td>374</td>
<td>72.9</td>
<td>87.2</td>
</tr>
<tr>
<td>1.025–1.030</td>
<td>243</td>
<td>70.0</td>
<td>82.7</td>
</tr>
</tbody>
</table>

### Table 3. Interference of Various Antibiotics on LEUKOSTIX Reagent-Strip Performance (Visual Analyses)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Conc (g/L) at which effect was observed</th>
<th>Degree and type of effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalexin</td>
<td>1.0</td>
<td>Moderate inhibition</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>0.8</td>
<td>Substantial inhibition</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>0.07</td>
<td>Atypical color</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.5</td>
<td>Substantial inhibition</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>0.6</td>
<td>Slight inhibition</td>
</tr>
</tbody>
</table>

*slight: barely detectable loss of reactivity at "trace" level; moderate: up to ½ color block loss; substantial: ¼ color block loss or more.

The following antibiotics did not significantly interfere with performance: trimethoprim, carbenicillin, ticarcillin, kanamycin, ampicillin, amoxicillin, penicillin G, gentamicin, colistin, streptomycin, ethambutol, nalidixic acid.
A Linear Method for Determining Specific Activity of Tracers in Radioimmunoassays

C. S. Chiang

I describe a rapid and objective method for graphically determining specific activity of radioactive tracers by self-displacement. Two sets of results are plotted on the same graph: total/bound counts vs (a) concentration of unlabeled analyte (for the standard curve results) or (b) quantity of radioactivity (for tracer self-displacement results). Because these plots are linear, not only are the difficulties of curve fitting and the need for numerous data points avoided, but also how well the data fit the lines can be assessed by calculating the standard deviation of the slope of the lines. This method minimizes uncertainty between data points, allows easier interpolation, and often yields more precise results than do previously published procedures.

Most ligand binding assays, including radioimmunoassays, require the use of a fixed concentration of tracer (label) to keep assay performance optimal (1, 2). This is especially important in assays where high sensitivity is required and iodinated tracers are used. Because of the short half-life of 125I, new tracers must be prepared at least every two months. Tracer concentration cannot be deduced simply from the amount of radioactivity present; therefore, determination of specific radioactivity is necessary for assessing the quality of the tracer. The most widely used procedure for estimating specific activity of tracers is self-displacement (3, 4).

These procedures are applicable to assays where the binder is relatively homogeneous and binds ligand (unlabeled analyte) and tracer with equal affinity. Although satisfactory for most applications, the published self-displacement procedures (3, 4) involve plots of complex curves to generate results, thus necessitating the use of subjective manual curve-fitting or complex curve-fitting computer programs. Here I describe a graphical method involving only linear plots for determining specific activity of tracers by self-displacement. This method can be easily computerized by using linear regression algorithms only. It is based on the finding that, in ideal binding reactions, the plot of total/bound counts (T/B) vs ligand concentration is linear (5, 6).

Materials and Methods

L-3,3',5'-Triiodothyronine ("reverse" T3, rT3) and [125I]rT3 were obtained from Calbiochem (Behring Diagnostics) and New England Nuclear, respectively. Antibody to rT3 was produced in a rabbit by immunizing the animal with a conjugate of rT3-bovine serum albumin monthly for six months. Barbitral, bovine serum albumin, and Polyethylene Glycol 6000 were purchased from Sigma, Miles, and Eastman Kodak, respectively. Antibody, rT3, and [125I]rT3 were incubated in barbital buffer (50 mmol/L, pH 8.6) at 20 °C for 3 h (0.4 mL total volume). Bound and free radioactivity were separated by adding Polyethylene Glycol 6000, centrifuging at 2000 × g for 20 min and aspirating the supernatant, which contains the free fraction.

For determining the tracer's specific activity, I used two sets of tubes. The tubes in the first set contained constant quantities of antibody and tracer, with increasing amounts of unlabeled analyte. The tubes in the second set contained the identical quantity of antibody but increasing amounts of tracer; the unlabeled analyte was omitted. I plotted the two sets of results on the same graph: total/bound counts (T/B) vs concentration of unlabeled analyte, and T/B vs quantity of tracer (radioactivity).

Results and Discussion

To best illustrate the utility of this method, I present an...