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Spectrophotometric Determination of Leukocytes in Urine

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A spectrophotometric method based on myeloperoxidase activity for the determination of leukocytes in urine is described. Red cells that may be found in urine samples were lysed by an ammonium chloride method. Leukocytes were then sedimented by centrifugation and lysed using Triton X-100 (Sigma Chemicals Co., St. Louis, MO). Myeloperoxidase-catalyzed oxidation of o-dianisidine was carried out at 37°C, pH 7. The reaction was stopped with the addition of 2 M H_2SO_4, and a stable form of oxidized o-dianisidine in acidic solution was obtained. Solid particles that may be found in urine samples were removed by centrifugation to avoid turbidity, and absorbance values of the supernatants were recorded at 400 nm. An Average number of leukocytes were noted per number of fields by microscopic examination and were related with the absorbance values of the supernatants at 400 nm. Pearson correlation (r) between our presented spectrophotometric analysis results and visual microscopic analysis was 0.877. Roche Combur 10-test M strips (Roche, Mannheim, Germany) and Multistix 10 SG Bayer test strips (Bayer Diagnostics, UK) were 0.645 and 0.648, respectively ($P < 0.0001$). J. Clin. Lab. Anal. 18:251–254, 2004. © 2004 Wiley-Liss, Inc.

Key words: leukocyte; determination; urine; spectrophotometry; o-dianisidine

INTRODUCTION

The presence of leukocytes in the urine is an important sign of inflammation. Most clinical laboratories detect leukocyturia by directly examining the urinary sediment. For many reasons, however, routine urinary microscopic examination is being questioned as a method of screening for urinary tract disease. Urine test strips based on esterase or elastase activity, are generally used for visual inspection. These strips present the results in an ordinal scale for the rough quantitative determination of leukocytes in urine. Considerations of economics, efficiency, and staffing have caused many clinical laboratories to seek new alternative methods for urine analysis (1–5).

The antimicrobial system found in leukocytes includes peroxidase enzymes, hydrogen peroxide, and halide or thiocyanate ions. The antimicrobial activity of myeloperoxidase has been related to the chloride peroxidase activity of the enzyme. Human neutrophils and monocytes generate hydroxyl radicals through a myeloperoxidase-dependent mechanism (6–12). Singlet oxygen is a product of several peroxidase-hydrogen peroxide-halide systems (13,14). Eosinophil peroxidase oxidizes chloride ions less efficiently than does myeloperoxidase, and it preferentially oxidizes bromide in the presence of at least a 1,000-fold excess of chloride (15–17).

B lymphocytes and B-lymphotic cell lines, together, express a 5-lipoxygenase protein. The selenium-dependent peroxidases are responsible for the suppression of cellular 5-lipoxygenase activity in B lymphocytes and immature myeloid cells. B cells can be considered as a source to induce leukotriene synthesis under oxidative conditions, and therefore support the inflammatory process (18,19). The intracellular myeloperoxidase of lymphocytes has been demonstrated by flow cytometric analysis and cytoplasmic staining methods (20,21). The regulation of cytokine biosynthesis in the cells and signaling is redox dependent. However, T lymphocytes are the specific source of interleukin-4 and interferon gamma (22–24).

Spectrophotometric methods based on the oxidation of chromogen substrates have been reported for the measurement of myeloperoxidase activity (25). Poly-

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morphonuclear leukocyte numbers in tissues have been determined by detecting peroxidase activities (26,27). Recently, we described a method for determining leukocyte concentration in human blood by detecting the stable form of oxidized o-dianisidine in acidic solutions. o-Dianisidine has been oxidized by redox enzymes found in leukocytes (28). In this study, this method was modified and adapted for determining leukocyte concentration in human urine samples.

MATERIALS AND METHODS

Materials

Triton X-100 and o-dianisidine were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Other chemicals used were all of analytical grade.

o-dianisidine solution

An amount of 0.2 Millimole (0.2443 g) o-dianisidine was dissolved in about 10 mL distilled water by the addition of 0.2 mM HCl. The volume was adjusted to 20 mL with water and 10 mM o-dianisidine solution was obtained (25,28).

Methods

Microscopic counting, isolation, and lysis of leukocytes

We studied 62 freshly collected urine samples submitted to the hospital for diagnostic urinalysis between 9:00 and 10:30 am. The samples were kept in an air-conditioned room at 20°C until assayed. All samples were completely processed within 1–2 hr after arrival (3).

Ten-milliliter urine samples were sedimented by centrifuging for 3 min at 1,500 rpm. After decanting the supernatant, we thoroughly mixed the sediment and placed a drop of it on a glass slide and applied a coverslip. We examined the contents of 10–15 microscopic fields at $40\times$ high power field and averaged the number of formed elements that were noted per number of areas (1). Leukocyte analysis was carried out simultaneously using Roche Combur 10-test M strips (Roche, Mannheim, Germany) with the Miditron Junior II (Boehringer Mannheim) and using Multistix 10 SG Bayer test strips.

At around 11:00 am, 10 mL of each urine sample was transferred to 15-mL polystyrene tubes, and 1 mL of 10-fold concentrated ammonium chloride lysis solution was added to the urine samples for the lysis of red cells that may be found in urine samples. Tube contents were incubated for 10 min at 20°C. Leukocytes were pelleted by centrifugation using the same conditions mentioned above and supernatants were decanted. An amount of 50 μL 0.2% (v/v) Triton X-100 was pipetted into the tubes, mixed by vortex, and incubated for 5 min at room temperature for the lysis of leukocytes found in pellets (26,28,29).

Spectrophotometric Assay

An amount of 840 μL 10 mM PBS buffer pH 7, containing 0.9% NaCl incubated at 37°C in a water bath, was added to the lysed leukocytes in tubes and mixed by vortex. An amount of 40 μL 10 mM o-dianisidine was added and tubes were placed in a water bath at 37°C. The reaction was started with the addition of 10 μL 3% H_2O_2. After 10 min, the reaction was stopped by the addition of 60 μL 2 M H_2SO_4, and a stable reaction product giving λ_{max} at 400 nm was obtained (25–28). Solid particles that may be found in urine samples pelleted together with leukocytes were removed by centrifugation for 5 min at 2,500 rpm. The supernatants were pipetted into the cells and absorbances at 400 nm were recorded using a Unicam (UK) model UV2, UV/V is spectrophotometer (28).

Statistics

Agreement between spectrophotometric results and visual microscopic analysis, Roche Combur 10-test M strips, and Multistix 10 SG Bayer test strips (Bayer Diagnostics, UK) was evaluated with Pearson regression analysis using Microsoft Excel, version 6 (Microsoft Co., Redmond, WA).

RESULTS AND DISCUSSION

Recently, we observed that the oxidized o-dianisidine obtained by the catalysis of redox enzymes found in leukocytes was very stable in acidic solution since the daily recorded absorption spectra did not change for 1 week. Hence, the absorption maximum of the oxidized substrate appearing at 400 nm was utilized for the determination of leukocyte concentration in blood (28).

In this study, red cells that may be found in urine were eliminated by ammonium chloride lysis before the centrifugation of urine samples. The oxidation of o-dianisidine was performed at 37°C instead of 25°C, using a more concentrated reagent solution (28). The reaction was stopped with the addition of 2 M H_2SO_4, and the observed turbidity in some samples was removed by centrifugation for 5 min at 2,500 rpm.

Methods used for the quantitative assessment of leukocytes in urine generally give ordinal scaled results (30). Similarly, as shown in Table 1, leukocyte numbers determined by microscopic examination and the corresponding absorbancies recorded for 10 mL urine samples were compared. Absorbances (400 nm) vs. leukocytes/high-power field are presented in Fig. 1.

Table 1. Leukocyte number and corresponding absorbance of urine samples at 400 nm

Leukocytes high-power field	Absorbance (400 nm)
0–2	0–0.1
2–5	0.1–0.5
5–15	0.15–0.2
15–30	0.2–0.4
30–50	0.4–0.6
50<	0.6<

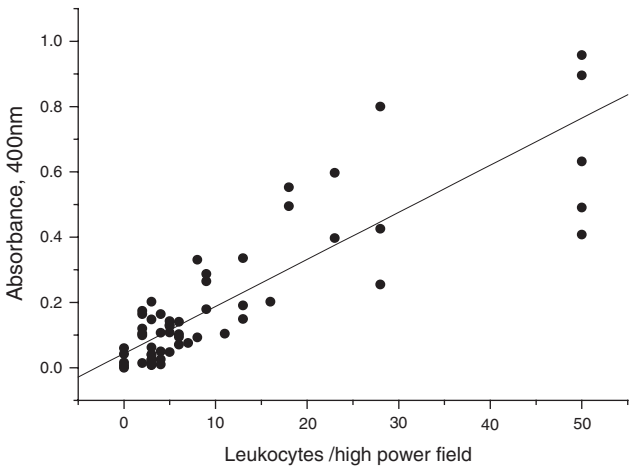

Fig. 1. The absorbances corresponding to the "leukocytes/high power field" of microscopic sediment examination. (Linear regression: $y = 0.044 + 0.014x$, $R = 0.877$, $SD = 0.118$, $n = 62$, $P < 0.0001$).

The correlation (r) between our spectrophotometric analysis results and visual microscopic analysis was 0.877 and that of Roche Combur 10-test M strips (Roche, Mannheim, Germany) and Multistix 10 SG Bayer test strips (Bayer Diagnostics, UK) were 0.645 and 0.648, respectively. Our spectrophotometric method agrees well with microscopic leokocyte counts and the above mentioned semi-quantative commercial test strips.

Comparison of the sensitivities, specificities, and costs of the methods used for the detection of leukocytes in urine gives rise to many questions (1–3,5). Hence, the detection of leukocytes in urine samples, using a stable reaction product of myeloperoxidase catalysis, is a contribution to this field. Our method is reliable and inexpensive. In addition, measured absorbances are proportional to the number of leukocytes present in the urine samples.

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