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A new screening method for proteinuria using Erythrosin B and an automated analyzer—Rapid, sensitive and inexpensive determination

Satoshi Horikoshi [a], Asami Higurashi [b], Emiko Kaneko [c], Hajime Yoshimura [b], Isao Ohsawa [a], Yusuke Suzuki [a], Chieko Hamada [a], Yasuhiko Tomino [a,*]

[a] Division of Nephrology, Department of Internal Medicine, Juntendo University Faculty of Medicine, Tokyo, Japan
[b] Shino-Test Co., 2-29-14 Oonodai Minami Sagamihara Kanagawa 252-0203, Japan
[c] New Industry Creation Hatchery Center, Tohoku University, Sendai 980-8579, Japan

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ABSTRACT

Background: In spite of the urgent necessity for a screening test of urinary protein for the early diagnosis of kidney diseases, a rapid, accurate and cost-effective method for their detection has yet to be developed.
Methods: A solution containing a buffer agent (pH 2.3) and surfactants and a solution of Erythrosin B are added to a urine sample. After letting the mixture stand for 5 min at 37 °C, the dye-bound protein is measured by a spectrophotometer at 546 nm using a Hitachi 7170S automated analyzer.
Results: The calibration curve was linear with human serum albumin concentration in the range of 2.4–200 mg/l. The detection limit, 2.4 mg/l was superior to conventional dye-binding methods by one order of magnitude and comparable to the turbidimetric immunoassay (TIA). Spot urine samples from 70 patients who showed (−) or (±) in the dip-stick screening test for proteinuria and 79 healthy volunteers were analyzed. There was an excellent correlation ($r = 0.978$, $n = 149$) between the results given by the proposed method and those by the TIA.
Conclusions: This method provides a viable alternative to the conventional immunoassay-based methods for urinary protein measurement, and will be useful in the diagnosis of early stage kidney disease.

1. Introduction

Urine sample collection is noninvasive and pain free, and the samples provide a lot of important biological information, which can be used in the diagnosis of a wide range of medical conditions. Our particular interest is focused on the detection of urinary proteins, typically albumin, which is essential for the detection of renal diseases. In recent years, the number of patients with end-stage kidney disease (ESKD) requiring dialysis or transplantation has markedly increased worldwide [1–4]. In Japan, about ten thousand patients undergo dialysis therapy every year and the number with estimated glomerular filtration rate (eGFR) of <60 ml/min who are at risk of progression to ESKD and cardio- and cerebrovascular diseases has already exceeded twelve million [5,6]. This alarming number of renal patients will surely be a heavy burden on the medical system, especially in terms of cost. Parallel to the increase in patients with diabetes is the rise in the incidence of diabetic nephropathy, which is also the most frequent underlying disease of ESKD, and ESKD patients should increase further in the future. In addition, chronic kidney disease (CKD), which is precondition of ESKD and cardiovascular disease, is characterized by chronic persistence of findings suggestive of renal disorder or decreased renal function such as eGFR<60 ml/min [7–9]. When everything is taken into consideration, it is clear that establishing a method for detecting CKD is an issue of global importance. The early detection of CKD is important because its progression can be suppressed and remission is common if therapeutic intervention is performed earlier rather than later.

Various biomarkers have been investigated extensively for early diagnosis of kidney disease, such as Neutrophil gelatinase-associated lipocalin (NGAL). However, their application to early diagnosis is extremely limited. The object of our study is to develop a rapid, simple, and inexpensive analytical method, with particular focus on urinary albumin, which is well recognized as an effective marker of early stage kidney disease and cardiovascular disease. The detection of urinary albumin, which appears in urine from an early stage of the disease, and the amount of albuminuria are strong predictors for decline of renal function in the late stage of CKD [10–13]. Since measurement of urinary albumin in the general population is not cost-effective [14,15], the most common urinary protein test currently in use is a semi-quantitative test with urine dipsticks, used not only for

Abbreviations: PR-Mo, Pyrogallol Red-molybdenum (PR-Mo) method; TIA, turbidimetric immunoassay; ESKD, end-stage kidney disease; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; JCCLS, Japanese Committee for Clinical Laboratory Standards; THP, Tamm–Horsfall protein.

* Corresponding author at: Division of Nephrology, Department of Internal Medicine, Juntendo University Faculty of Medicine, 2-1-1 Hongo, Bunkyou-ku, Tokyo 113-8421, Japan. Tel.: +81 3 5802 1065; fax: +81 3 3813 1183.
E-mail address: yasu@juntendo.ac.jp (Y. Tomino).

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one-on-one testing but also for mass screening [16,17]. However, it is not sufficiently sensitive to detect microalbuminuria, and this simple method often fails to identify patients with early stage CKD [18]. The Pyrogallol Red-molybdenum (PR-Mo) method [19], which is one of the most frequently used methods for urinary protein determination in the follow-up testing of patients based on dye-binding spectrophotometry using PR-Mo complex, also has insufficient detection sensitivity (lower detection limit: 37 mg/l). Turbidimetric immunoassay (TIA) is sufficiently sensitive, with a lower detection limit of 1 mg/l, but its application is limited because the reagent is prohibitively expensive. The highly sensitive method we have developed for testing urinary protein levels is based on a dye-binding method using Erythrosin B (Acid Red 51). Soedjak was the first to show the effectiveness of using Erythrosin B in the spectrophotometric determination of protein in aqueous solution at levels as low as 2 mg/l [20]. Her method, which uses a dye binding reaction at 90–95 °C, has been improved upon over the years. The detection limit has been significantly improved to levels as low as 0.06 mg/l at room temperature [21], making it possible to develop a simple yet sensitive visual spot test for urinary protein [22]. However, neither the achievement of high sensitivity nor the availability of a spot test has made the dye-binding method suitable for mass screening: hands on, time-consuming work is still required to prepare the samples prior to testing. Moreover, the measurable ranges were too narrow to be applied to urine samples. The need for the development of an automated analyzer in order to make the test suitable for routine use in the clinical laboratory is clear. By adding an automated analyzer, the high sensitivity screening of urinary protein is possible with the simultaneous determination of substances such as creatinine. This low-cost method is not only a rapid and simple alternative to TIA, but it is also capable of a high throughput of more than 1000 samples in one working day. In the present study, we applied the Erythrosin B dye-binding method coupled with an automated analyzer and developed a new method for measurement of multiple samples within a short time. Urinary protein was measured in spot urine samples obtained from patients with diabetes, hypertension or CKD, and healthy volunteers. A comparison of the results obtained by our method and those by more established techniques, PR-Mo and TIA, is included.

2. Materials and methods

2.1. Subjects

Seventy outpatients in the Division of Nephrology and Hypertension of Juntendo University Hospital who were (−) or (±) in the dipstick test screening for proteinuria were enrolled in this study. Spot urine samples from the patients and 79 healthy volunteers were examined. The study protocol was approved by the institutional review board of Juntendo University Hospital and all patients gave written informed consent. Urine samples were stored at −80 °C.

2.2. Reagents

This method is a 2-reagent system; the first reagent consists of a buffer solution and surfactants, and the second one consists of a dye solution. TritonX-100 (Nacalai Tesque, Inc., Japan) and 0.45 g of TritonX-405 (Nacalai Tesque, Inc., Japan) were dissolved in water; 4 mol/l NaOH was added dropwise to achieve pH 2.3; and, pure water was added to make a total volume of 1 L.

Reagent II (R-II): 0.1759 g of Erythrosin B (Wako Pure Chemical Industries Ltd., Japan) and 0.6057 g of Tris-[hydroxymethyl]aminomethane (TRIS) (MP Biomedicals, Inc.USA) were dissolved in water; 1 mol/l HCl was added dropwise to achieve pH 7.5; and, pure water was added to make a total volume of 1 l. After preparation, each test solution was kept refrigerated in a light-resistant bottle.

Human serum albumin (Fraction V; Wako), γ-globulins (from human blood), β_2-microglobulin (from human urine), retinol binding protein (from human urine), α_1-acid glycoprotein (from human plasma), α_1-antitrypsin (from human plasma), Transferrin (human) (Sigma. Co. St. Louis, MO), α_1-microglobulin (human) (AbD Serotec, UK), Bence Jones protein (κ, λ) (human) (Nordic Immunological Labs., Netherlands) and Tamm–Horsfall protein (human) (Harbor Bio-Products Inc. Norwood, MA) as proteins potentially present in urine, were also used to evaluate reactivity.

Lyophilized products were used after being dissolved in physiological saline. For cross validation, Micro TP-test WAKO (Wako Redmond, VA) based on the PR-Mo method, and Auto Wako Microalbumin (Wako) test based on TIA, were used.

2.3. Instrumentation

For measurement, an automatic analyzer 7170S (Hitachi Ltd., Japan) with a throughput of 800 tests per hour was used. In addition, spectrophotometric measurement was performed using a V-650 spectrophotometer (JASCO Corporation, Japan).

2.4. Procedure for automated assay

Before the assay, urine samples were thawed on ice and incubated in water bath. At 37 °C, 150 μl of R-I was mixed with 6 μl of urine sample; 30 μl of R-II was mixed with the solution 5 min later, and a color reaction was developed for 5 min. Absorbance determined by the following equation was used: absorbance after the end of the second reaction–absorbance at the end of the first reaction. A 2-wavelength analysis was performed at a main wavelength of 546 nm and a sub-wavelength of 700 nm. For calibration, the absorbance of albumin-physiological saline solution (40 mg/l) was used.

3. Results

3.1. Optimization of analytical conditions

The analytical procedure was optimized with respect to the pH value, solution composition and concentration of Erythrosin B. It is well recognized that interaction between a dye and a protein is strongly dependent on pH. Anionic dye binds to the positively charged site of protein molecules under acidic conditions through electrostatic and hydrophobic interactions. In our previous work, the acid dissociation constant of Erythrosin B was determined as 3.20 for pK_{a1} [22].

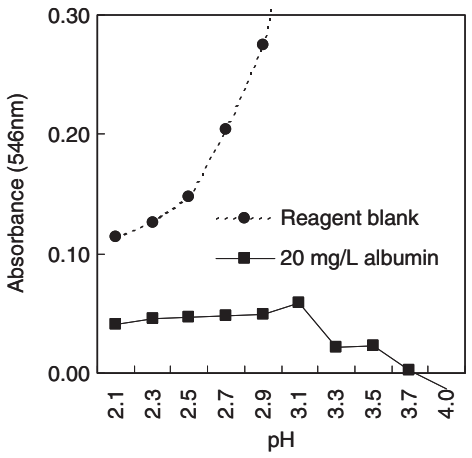

Fig. 1. Effect of pH on absorbance of reagent blank using saline samples without albumin (●) and net absorbance for 20 mg/l albumin (■). pH buffer agent: 0.17 mol/l citric acid — NaOH; surfactants: 0.21 g/l TritonX-100 and 0.33 g/l TritonX-405; Erythrosin B: 33 μmol/l.