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Successive determination of urinary bilirubin and creatinine employing simultaneous injection effective mixing flow analysis

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ABSTRACT

A novel four-channel simultaneous injection effective mixing flow analysis (SIEMA) system has been assembled for successive determination of bilirubin and creatinine in urinary samples. The chemical variables and physical parameters in the flow system were optimized for the enhancement of successive analytical performances. The interferences from urine matrices on the determination of bilirubin and creatinine were eliminated to dilute urine samples. The calibration graphs with the optimum conditions were achieved to be in 0.024–5.0 mg L^{-1} for bilirubin and 2–100 mg L^{-1} for creatinine. The relative standard deviations (RSDs) at 3 mg L^{-1} of bilirubin and at 50 mg L^{-1} of creatinine for 11 runs were 1.5 and 1.0%, respectively. The limits of detections (3σ of blank) for bilirubin and creatinine were 7 μg L^{-1} and 0.6 mg L^{-1}, respectively. The sample throughput for stepwise detection was 22 h^{-1}. The proposed method was applied to the successive determination of bilirubin and creatinine in urine samples.
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1. Introduction

Urine analysis (urinalysis) has been firmly established as a quick, inexpensive, and invaluable diagnostic tool for measurement of urologic conditions such as calculi, urinary tract infection (UTI), and malignancy [1–3]. It also can alert the physician to the presence of systemic disease affecting the kidneys. Urinalysis involves physical, chemical and microscopic analysis of the urine. The target parameters to mention the quality of urine are glucose, albumin, and trace metals, enzymes, blood cells and other molecules such as bilirubin and urobilinogen.

Bilirubin is the yellow breakdown product of the haem moiety of hemoglobin and other haemoproteins [4]. In the liver disease, hepatitis and blocked bile duct, bilirubin (especially conjugated bilirubin and/or direct bilirubin) is filtered by the kidneys. On the other hand, the evaluation is needed for liver dysfunction and biliary obstruction when it is detected in the urine [1,5,6]. The upper normal level of urine bilirubin is set at 1 mg L^{-1} [7]. Usually, batch-wise methods using color development with diazonium salt and spectrophotometry were proposed [5,8–10] for the determination of serum bilirubin and urine bilirubin [6], however, these methods are time-consuming and tedious.

Creatinine is a breakdown product of creatinine phosphate with muscle metabolism. It is produced in the body at a constant rate and is excreted through urine in small amounts [11]. Creatinine is removed from the body entirely by the kidneys. Creatinine level in blood and urine may be utilized to calculate the creatinine clearance which reflects the glomerular filtration rate (GFR) that is clinically important for renal function [12]. The rate of muscle metabolism slows down with age, and so, elderly people can have low urine creatinine levels. A 24 h sample test is often relied upon, to find out the urine creatinine levels. The normal creatinine level can be anywhere between 500 and 2000 mg/day [13]. Regularly, creatinine reacts with picric acid in an alkaline medium to form a red color compound [14]. However, this method is a batch-wise procedure that requires a large amount of reagents and/or sample, long time analysis and tiresome. Moreover, on Jaffé batch-wise reaction with alkaline picrate, 30 min standing time is needed to complete the color development at room temperature [14–16].

Flow based systems with many configurations as flow injection (FI) [17–24], sequential injection (SI) [25,26] and multicommutated flow analysis system (MCFA) [27] have been reported for the automated measurement of bilirubin [17–20] and creatinine [21–27]. However, to the best of our knowledge, application of

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flow based system in color based reaction has not been investigated for the successive determination of bilirubin and creatinine.

A spectrophotometric SI system has been developed for successive determination of urinary protein and glucose [28]. In addition, previous SI methods have played important roles in the automation and miniaturization of analytical methods. In a SI format, mutual penetration of sample and reagent(s) zones is essential for a successful chemical reaction. However it is usually difficult to obtain a well-mixed condition of these zones aspirated into a common holding coil. This lower mixing efficiency plagues the utilization of SI in some cases. The flow reversal was also employed in SI system to enhance the efficiency of mixing of analyte and reagents.

An alternative flow system in the name of simultaneous injection effective mixing flow analysis (SIEMA) has been developed to overcome the disadvantages of SI as a tool for rapidity, small reagent consumption, reproducibility and sensitivity. The novel concept of this technique has been reported for quantification of palladium [29], urinary bilirubin [5], urinary protein [30], residual chlorine in tap water [31] and bilirubin and urobilinogen in urine samples [32]. Apparently, the main advantages of SIEMA system in the analytical procedure are effective mixing of the chemical reaction, reduction of waste generation due to small amount of reagents and/or sample simultaneously aspirated and dispensed by syringe pump, fast analysis and fully automated operation. Although we assembled a four-channel SIEMA system for the successive determination of bilirubin and urobilinogen in a previous paper [32], the SIEMA system has not been applied to any other couples of analytes. In this study, a 4-channel SIEMA system applied to the successive spectrophotometric determination of bilirubin and creatinine in urine is demonstrated.

2. Experimental

2.1. Reagents and chemicals

All chemicals and reagents in this work were of analytical grade and were used without further purification. The water was purified by an Advantec GSH-210 apparatus and was utilized throughout the experiments.

A stock standard solution of bilirubin (50 mg L^{-1}) was prepared by dissolving 1.0 mg of ditaurate bilirubin (Promega, Madison WI, USA) in water to make up a volume of 20 mL. After that, the solution was kept in amber glass bottle for protection of photo-oxidation process of bilirubin and kept at −80 °C refrigerator.

A stock solution of sulfanilic acid (80 mmol L^{-1}) was generated by dissolving 0.6962 g of sulfanilic acid (Wako Pure Chemical Co., Japan) in 0.3 mol L^{-1} sulfuric acid to have a volume of 50 mL.

Sodium nitrite stock solution (200 mmol L^{-1}) was made by dissolving 0.2774 g of sodium nitrite (Wako Pure Chemical Co., Japan) in water and the volume was adjusted to 20 mL.

Stock solution of OTG 1.0% (w/v) was prepared by dissolving *n*-octyl-*β*-D-thioglucoside (Dojindo, Japan) in water.

The fresh working solutions of diazotized sulfanilic acid miscible with OTG were prepared by mixing appropriate volumes of 80 mmol L^{-1} of sulfanilic acid solution, 200 mmol L^{-1} sodium nitrite solution and together with 1.0% (w/v) of OTG. After that the solution was diluted to 10 mL with water.

Creatinine standard stock solution (1000 mg L^{-1}) was produced by dissolving 25 mg of creatinine (Wako Pure Chemical Co., Japan) in 0.1 mol L^{-1} HCl. The final volume was made up to 25 mL. Working standard creatinine was diluted in water.

100 mmol L^{-1} picric acid solution was generated by dissolving 2.691 g of picric acid (Wako Pure Chemical Co., Japan) in 100 mL of water.

10% (w/v) of sodium hydroxide was made from dissolving 10 g of sodium hydroxide (Nacalai Tesque, Inc., Japan) in 100 mL water.

The alkaline pricrate working solution was created by diluting suitable volume of 100 mmol L^{-1} pricric acid and 10% (w/v) NaOH. The fresh reagent prepared daily.

2.2. SIEMA set up

The four-channels of SIEMA system for successive quantification of bilirubin and creatinine in urinary samples is shown in Fig. 1. It was comprised of bidirectional syringe pump (5000 μL, CAVRO, USA) which used to aspirate/dispense whole of solution in the system. Four solenoid valves ($3V_1$, $3V_2$, $3V_3$ and $3V_4$) (Takasogo Electric, Japan) were employed to select each reagent. Other valve ($3V_5$) was used as similar as 2-way valve. It was closed when sample/reagent was aspirated into the syringe, and it was opened when the zone was delivered to detector. Teflon tubing (0.8 mm i.d.) was utilized as flow lines. A visible spectrophotometer (Soma Optics, S-3250, Japan) was operated for continuous monitoring OTG-azobilirubin and creatinine-picrate complex at 535 nm. The SIA MPV-SPV ver. 3.00b (M&G Chematex Japan, Japan) was employed for full automatic control and Chromato-PRO (Runtime Instruments, Japan) was used for data acquisition.

2.3. Analytical procedures

The stepwise of analytical procedures for successive bilirubin and creatinine determination are displayed in Table 1. The operation was started in bilirubin detection by A) simultaneous aspiration of

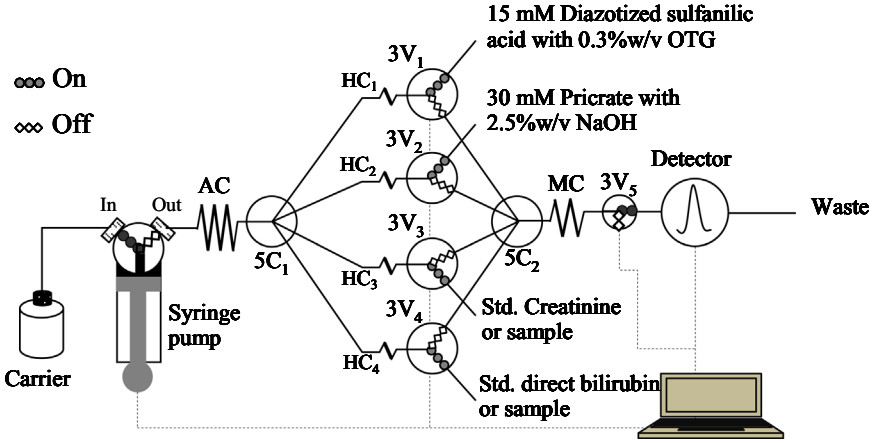

Fig. 1. The SIEMA system for successive urinary bilirubin and creatinine detection; AC, auxiliary coil (100 cm, 1.5 mm i.d.); $5C_1$ and $5C_2$, 5-way cross connectors; HC_1, HC_2, HC_3 and HC_4, holding coils (100 cm, 0.8 mm i.d.); $3V_1$, $3V_2$, $3V_3$, $3V_4$ and $3V_5$, 3-way solenoid valves; MC, mixing coil (100 cm, 0.8 mm i.d.).

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