



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



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ARTICLE INFO

Article history:

Received 21 January 2014

Received in revised form

11 May 2014

Accepted 26 May 2014

Available online 8 July 2014

Keywords:

Simultaneous injection effective mixing flow analysis

Bilirubin

Creatinine

Urinalysis

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 (3 σ of blank) for bilirubin and creatinine were 7 μ g 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 picrate working solution was created by diluting suitable volume of 100 mmol L^{-1} picric 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 $3V_4$) (Takasago 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

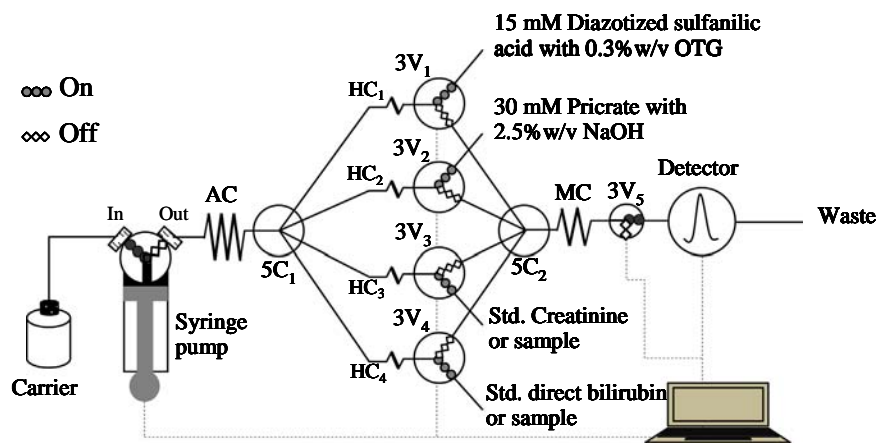


Fig. 1. The SIEMA system for successive urinary bilirubin and creatinine detection; AC, auxiliary coil (100 cm, 1.5 mm i.d.); $5C_1$ and $5C_2$, 5-way cross connectors; HC_1 , HC_2 , HC_3 and HC_4 , holding coils (100 cm, 0.8 mm i.d.); $3V_1$, $3V_2$, $3V_3$, $3V_4$ and $3V_5$, 3-way solenoid valves;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

Table 1

The protocol for the successive determination of bilirubin and creatinine in urinary samples.

Steps	Valve of syringe pump [↓↑] ^b	V ₁	V ₂	V ₃	V ₄	V ₅	Flow rate (μL s ⁻¹)	Volume (μL)	Operations
Bilirubin determination									
A	[↓] Off	On	Off	Off	On	Off	200	500 ^a	Simultaneous aspiration diazotized sulfanilic acid mixing with OTG, and std. bilirubin/sample to HC ₁ , and HC ₄
B	[↓] On	Off	Off	Off	Off	Off	750	4500	Aspiration carrier solution to syringe
C	[↑] Off	Off	Off	Off	Off	On	100	5000	Dispensing all aspirated zones to detector simultaneously
Creatinine determination									
D	[↓] Off	Off	On	On	Off	Off	200	500 ^a	Simultaneous aspiration alkaline picrate solution, and std. creatinine/sample to HC ₂ , and HC ₃
E	[↓] On	Off	Off	Off	Off	Off	750	4500	Aspiration carrier solution to syringe
F	[↑] Off	Off	Off	Off	Off	On	50	5000	Dispensing all aspirated zones to detector simultaneously

^a Aspiration volume of each reagent was approximately 250 μL.^b [↓] downward direction, [↑] upward direction.

500 μL of diazotized sulfanilic acid with OTG and standard bilirubin/sample to each holding coil (HC₁ and HC₄). Then, B) a syringe pump was switched to valve in for aspirating carrier to syringe. After that, C) aspirated zones were concurrently pushed toward to a detector by reversed flow for monitoring OTG-azobilirubin product. Then, the creatinine measurement was followed by D) simultaneous aspiration of alkaline picrate solution and standard creatinine/sample into HC₂ and HC₃. Afterwards, E) 4500 μL of carrier was sucked to syringe. Finally, F) the solutions in holding coils (HC₂ and HC₃) were simultaneously dispensed to the detector by bidirectional syringe pump.

2.4. Vanadate oxidation method

A batch-wise vanadate oxidation assay [33] was used as a reference method to validate the amounts of urine bilirubin obtained by the SIEMA. Briefly, a 10 μL sample was transferred to a 280 μL of buffer solution at around pH 3, the mixture was incubated for 5 min, a 70 μL of vanadate solution was added, and the mixture was then incubated again for 5 min (bilirubin was oxidized by vanadium(V) to biliverdin). Before and after adding a vanadium solution, the specific absorbances of bilirubin at around 450 nm were measured.

2.5. Collection of urine samples and sample preparation

Urine samples were collected from normal subjects. Then all samples were stored at 4 °C. Before analysis, the samples were filtered by filter paper (Whatman#1) to remove small particles. Next, the filtered solution was diluted at least 5 and 100-folds with water for bilirubin and creatinine detection, respectively.

3. Results and discussion

3.1. Detection wavelength

The visible spectrophotometric detector (Soma Optics, S-3250, Japan) was utilized to measure both analytes in the proposed method. The azobilirubin and creatinine-picrate complexes show different maximum absorption wavelengths at 560 nm and 500 nm, respectively. Therefore, the appropriate detection wavelength was studied between 500 and 560 nm. It was found that the sufficient absorbance for bilirubin and creatinine was obtained at 535 nm, although the sensitivities loss due to this wavelength modification for bilirubin and creatinine were 14 and 68%, respectively. Hence, 535 nm was selected to stepwise determination of bilirubin and creatinine in urinary samples.

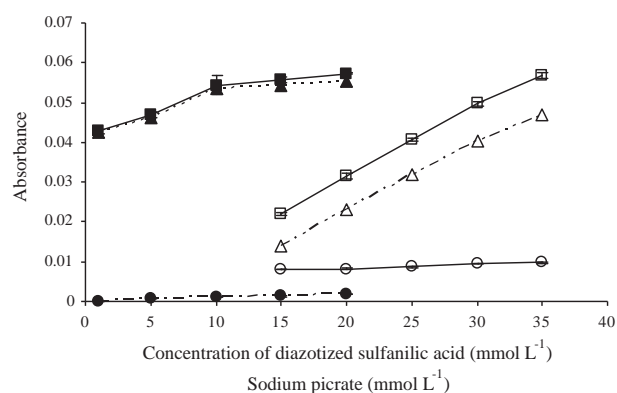


Fig. 2. Effect of diazotized sulfanilic acid and picric acid concentration; blank bilirubin (●), 5 mg L⁻¹ bilirubin (■), net signal bilirubin (▲), blank creatinine (○), 100 mg L⁻¹ creatinine (□), and net signal creatinine (△).

3.2. Optimization of variables for bilirubin and creatinine detection

3.2.1. Effect of diazotized sulfanilic acid and picric acid concentration

The concentrations of reagent for bilirubin determination as a diazotized sulfanilic acid were explored in the range of 1–20 mmol L⁻¹. The results are presented in Fig. 2. The sensitivity increased when the concentration of diazotized sulfanilic acid increasing up to 10 mmol L⁻¹ and the sensitivity was constant over 15 mmol L⁻¹. Therefore, diazotized sulfanilic acid concentration of 15 mmol L⁻¹ was selected for detection of bilirubin.

In the creatinine determination, sodium picrate was tested in the range 15–35 mmol L⁻¹ (Fig. 2). Absorbance also increased with increasing sodium picrate. However, over 35 mmol L⁻¹ of the picrate, the precipitation was observed. Therefore, 30 mmol L⁻¹ of sodium picrate was chosen for creatinine detection.

3.2.2. Effect of OTG and NaOH concentration

Matsudo et al. [34] added OTG to extract and separate bilirubin into surfactant-rich phase. To enhance the sensitivity, the effect of OTG was tested in the range 0–0.7% (w/v) (Fig. 3). It was found that absorbance increased with increasing OTG concentration until 0.45% and over this concentration, the sensitivity was almost constant. 0.45% (w/v) of OTG was chosen for the proposed method. It seems that OTG might serve as a stabilizing agent for azobilirubin [5].

The reaction of creatinine and sodium picrate was developed in the alkaline medium. In this work, sodium hydroxide was investigated to control the reaction condition in the range 1.5–3.0% (w/v). Absorbance increased with increasing sodium hydroxide concentration (Fig. 3). However, over 2.5% sodium hydroxide resulted in the precipitation of

sodium picrate. Hence, in this work, 2.5% (w/v) of sodium hydroxide was utilized for creatinine determination.

3.2.3. Effect of simultaneous aspiration volume

In this work, the effect of simultaneous aspiration volume was studied to minimize consumption of the whole reagents and standard/sample volume concurrently aspirated into each holding coil. Total aspiration volumes were studied between 300 and 600 μL in which each line was equally drawn (i.e. between 150 and 300 μL). It was found that a small aspiration volume gave a low sensitivity. On the other hand, absorbance was enhanced with increasing total aspiration volume. Aspiration volume over 500 μL offered higher sensitivity, however, a large amount of waste was generated. A 500 μL of concurrent total aspiration volume was chosen to save reagent and sample.

3.2.4. Effect of mixing coil length

Although the mixing of reagent and analyte is expected to begin at 5-cross connector ($5C_2$), the mixing coil length was varied in the range of 30–150 cm for effective mixing and enhancing sensitivity. The mixing coil length slightly affected the rapid reaction between bilirubin and diazotized sulfanilic acid on the bilirubin determination. On the other hand, in the reaction with creatinine and sodium picrate in alkaline, absorbance was also increased with increasing the mixing coil up to 100 cm. Over the length, absorbance was almost constant. A 100 cm of mixing coil was selected for our system.

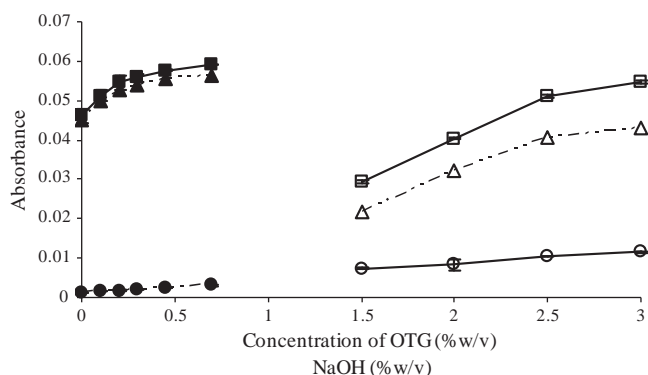


Fig. 3. Effect of OTG and NaOH concentration; blank bilirubin (\bullet), 5 mg L^{-1} bilirubin (\blacksquare), net signal bilirubin (\blacktriangle), blank creatinine (\circ), 100 mg L^{-1} creatinine (\square), and net signal creatinine (\triangle).

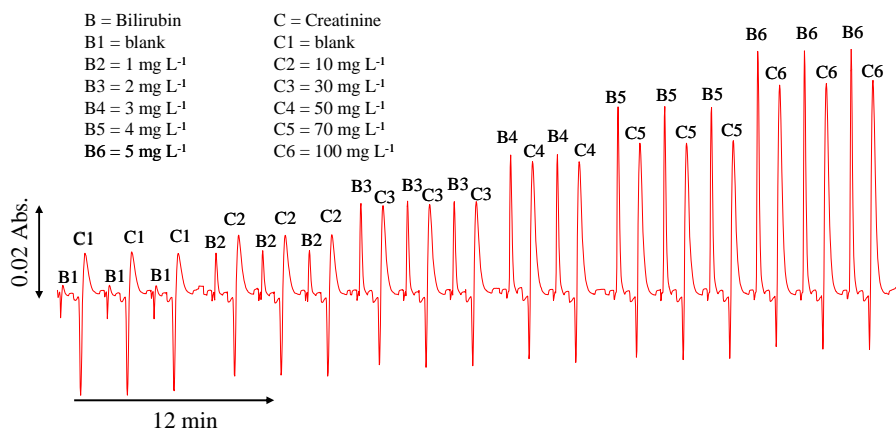


Fig. 4. The signal profiles obtained from our developed method.

3.2.5. Effect of dispensing flow rate

The effect of dispensing flow rate for delivering all of the aspirated zones to the detector was investigated because it can improve the sensitivity and frequency of analysis per hour. In the bilirubin determination, dispensing flow rate in the range of 50–150 $\mu\text{L s}^{-1}$ was studied. It was observed that the net signals were not significantly different because the reaction of bilirubin and diazotized reagent is rapid. So, the dispensing flow rate at 100 $\mu\text{L s}^{-1}$ was chosen for bilirubin determination. For creatinine determination, the flow rate was tested in the range 30–100 $\mu\text{L s}^{-1}$. It was found that utilizing low dispensing flow rate gave the higher sensitivity because of slow reaction time. However, 50 $\mu\text{L s}^{-1}$ was selected for the rapid determination of creatinine. Under the proposed dispensing flow rate, the sampling rate was 22 h^{-1} .

3.3. Analytical performances

Under the optimum conditions, the calibration graphs for successive determination of bilirubin and creatinine were excellently achieved between 0.024–5 mg L^{-1} and 2–100 mg L^{-1} respectively. The analytical signals are shown in Fig. 4. The linear equation for direct bilirubin is $y = (1.18 \times 10^{-2} \pm 7.95 \times 10^{-5}) C_B - (3.79 \times 10^{-3} \pm 2.63 \times 10^{-4})$, $r^2 = 0.997$ and that for creatinine, $y = (4.04 \times 10^{-4} \pm 1.11 \times 10^{-5}) C_C - (3.28 \times 10^{-4} \pm 6.78 \times 10^{-4})$, $r^2 = 0.998$, where y is absorbance and C_B and C_C are the concentration of bilirubin and creatinine, respectively in mg L^{-1} . The limits of detection defined as 3σ (σ as the standard deviation of blank ($n=3$)) for bilirubin and creatinine were 7 $\mu\text{g L}^{-1}$ and 0.6 mg L^{-1} , respectively. The limits of quantitation (10σ) of bilirubin and creatinine were 24 $\mu\text{g L}^{-1}$ and 2 mg L^{-1} . The RSDs at 3 mg L^{-1} of bilirubin and 50 mg L^{-1} of creatinine for 11 runs were 1.5 and 1.0%, respectively. Moreover, the developed system consumes minute volume of reagent as 500 μL per analysis.

3.4. Interferences study

The effect of foreign substances excreted into urine such as urea, chloride, and sulfate were investigated on the determination of bilirubin and creatinine by the proposed method. In our system, various chemical substances were spiked into 5 mg L^{-1} of bilirubin and 100 mg L^{-1} of creatinine for the tolerance limit within $\pm 5\%$ deviation. The results are summarized in Table 2. The mean concentrations of chloride, creatinine and urea in urine are 4780, 1960 and 18,200 mg L^{-1} [30,35]. From the results, it was found that the major foreign substances in urine such as chloride, creatinine and urea up to 1000 mg L^{-1} did not interfere after

samples were diluted 5-folds for bilirubin or 100-folds for creatinine before analysis.

3.5. Dilution effect and recovery

Since urine contains variable matrix that may influence the urinary bilirubin and creatinine determination, dilution was operated offline before measurement by the SIEMA system. The effect of dilution was investigated to certify accurate quantitation. Urine

sample may be diluted to eliminate coexisting substances that can cause negative or positive error on the determination. The real sample was diluted with water at various dilution factors (Table 3). And then, recovery of spiking the standard solution of 3 mg L⁻¹ bilirubin and 50 mg L⁻¹ creatinine was examined. It was found that 5 and 100 times of dilution ratio for bilirubin and creatinine could eliminate urine matrices. Therefore, 5 and 100 times of dilution factor were employed for successive determination of bilirubin and creatinine by the developed method.

3.6. Application

The proposed SIEMA system was applied to the determination of bilirubin and creatinine in 8 urine samples of healthy subjects. The samples were diluted at least 5 folds for bilirubin and 100 folds for creatinine. The results obtained by the proposed method were compared with a commercial vanadate oxidation method (See Section 2.4.). The results are summarized in Table 4. It was found that *t*-calculate values for bilirubin and creatinine were 1.02 and 0.48 respectively, which were less than *t*-critical (2.36) for 95% confidence level at 7 of degree of freedom. The relative errors of bilirubin and creatinine concentrations when comparing with the reference methods were found to be 1–35% and 0.6–8% for bilirubin and creatinine, respectively. It can be confirmed that the results of bilirubin and creatinine concentrations by the proposed SIEMA system agreed well with the results obtained by the conventional batch-wise vanadate oxidation and Jaffé reaction, with the linear equation: $y=0.825x+0.113$, $r=0.893$ for bilirubin and $y=1.01x+0.728$, $r=0.995$ for creatinine ($n=8$).

Table 2

Tolerance limits to foreign substances for successive bilirubin and creatinine determination.

Substances	Tolerance limit (mg L ⁻¹) (maximum concentration causes a deviation of ± 5%)	
	Bilirubin detection (5 mg L ⁻¹)	Creatinine detection (100 mg L ⁻¹)
Sodium chloride	500	4000 ^a
Potassium chloride	1000	4000 ^a
Ammonium chloride	1000	2000 ^a
Magnesium chloride	2000	500
Calcium chloride	850	200
Sodium sulfate	100 ^a	4000
Albumin	1000	5000 ^a
Ascorbic acid	1	5000 ^a
Glucose	4000 ^a	10000 ^a
Urea	5000	15000
Creatinine	2000	–
Bilirubin	–	5 ^a

^a maximum concentration tested.

Table 3

The recoveries of the successive determination of 3 mg L⁻¹ bilirubin and 50 mg L⁻¹ creatinine in a real urinary sample by different dilution factors.

Bilirubin assay		Creatinine assay	
Dilution factor	Recovery (n=3)	Dilution factor	Recovery (n=3)
0	89 ± 0.5	10	127 ± 1.1
2	92 ± 0.3	30	116 ± 1.0
5	97 ± 0.6	50	107 ± 0.9
10	101 ± 1.2	70	103 ± 0.5
15	104 ± 1.7	100	100 ± 1.1

n=number of experiments.

Table 4

The concentration of bilirubin and creatinine obtained by the present method and conventional vanadate oxidation method.

Samples	Bilirubin (mg L ⁻¹)		Relative errors (%)	Creatinine (mg L ⁻¹ , n=3)		Relative errors (%)
	SIEMA (n=3)	Vanadate oxidation (n=2)		SIEMA (n=3)	Jaffé batch-wise (n=2)	
1	0.45 ± 0.04	0.6 ± 0.1	–20	177 ± 17	183 ± 19	–3
2	1.35 ± 0.04	1.5 ± 0.1	–10	390 ± 39	360 ± 36	8
3	1.74 ± 0.20	2.5 ± 0.1	–29	449 ± 19	467 ± 14	–3
4	2.87 ± 0.03	2.5 ± 0.1	14	978 ± 31	993 ± 22	–1
5	1.41 ± 0.08	2.2 ± 0.2	–35	694 ± 27	641 ± 14	8
6	0.41 ± 0.10	0.3 ± 0.0	28	372 ± 21	397 ± 11	–6
7	1.86 ± 0.02	1.8 ± 0.1	–1	805 ± 44	792 ± 54	1
8	0.67 ± 0.03	0.5 ± 0.0	24	732 ± 10	727 ± 6	0.6

n=number of experiments.

4. Conclusion

A simple four-channel SIEMA system for successive of bilirubin and creatinine in urinary samples were prosperously developed. The color reactions utilized for the detections of bilirubin and creatinine were based on the diazo and Jaffé reactions. In the proposed SIEMA method, all parameters can be controlled by personal computer resulting in fully automated operation, short analysis time and minimized waste generation. The analytical results of bilirubin and creatinine in urine obtained by the proposed system were in good agreement with the bath-wise vanadate oxidation and Jaffé reaction. Therefore, we conclude that this newly developed SIEMA system should be alternative and effective for clinical routine assay for liver and kidney function test.

Acknowledgments

K.P. and K.G. acknowledge The Thailand Research Found (TRF) for the Royal Golden Jubilee Ph.D. program and Chiang Mai University through the Center of Excellence for Innovation in Analytical Science and Technology. N.T. acknowledges the financial support of this study by Grants-in-Aid for Scientific Research (KAKENHI) No. 26288072 from Japan Society for the Promotion of Science.

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