Talanta 87 (2011) 113–117

Contents lists available at SciVerse ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Simultaneous injection effective mixing analysis system for the determination of direct bilirubin in urinary samples

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ARTICLE INFO

Article history:
Received 13 July 2011
Received in revised form
19 September 2011
Accepted 19 September 2011
Available online 12 October 2011

Keywords:
Bilirubin
Simultaneous injection effective mixing analysis
n-Octyl-β-D-thioglucoside
Diazotized sulfanilic acid

ABSTRACT

A novel simultaneous injection effective mixing analysis system (SIEMA) for determination of direct bilirubin in urine sample was developed. Bilirubin reacts with diazotized sulfanilic acid in the presence of *n*-octyl-β-D-thioglucoside (OTG) as a solubilizing agent to form OTG-azobilirubin. The flow and chemical variables were investigated. A linear calibration graph for direct bilirubin was obtained over the range of 0–1.0 mg L^{-1} (r^2 = 0.994) with the limit of detection (3σ) of 4.7 μg L^{-1}, and the relative standard deviation (RSD) being 1.9% (n = 11, 0.5 mg L^{-1} of direct bilirubin). The results in healthy adult urine obtained by the proposed approach were found in good agreement with those obtained by the batch-wise diazo method.

1. Introduction

Bilirubin is the yellow breakdown product of the haem moiety of haemoglobin and other haemoproteins [1]. Bilirubin in serum has at least 4 distinct forms. α-Bilirubin called indirect fraction is the un-conjugated form (26% of total bilirubin), and it is soluble in alcohols. β- and γ-bilirubin fractions are mono- and di-conjugated with glucuronic acid, respectively (24% for mono-conjugated and 13% for di-conjugated). δ-Bilirubin is irreversibly bound to protein (37%). β-, γ- and δ-fractions are soluble in water, and they correspond to the direct bilirubin [2]. As bilirubin is excreted in serum and bile through the liver, bilirubin is usually not excreted into the urine, and higher levels of bilirubin in serum may indicate certain diseases, especially liver diseases [1]. It is responsible for the yellow color of bruises [3,4], urine and the yellow discoloration in jaundice [5,6]. Under the physiological condition, bilirubin is present in small amount in blood and urine. However, the bilirubin concentration in the blood increases when haemoglobin in the red blood is destroyed with liver disease and liver function disorder (hepatitis and blocked bile duct). Consequently, only conjugated form and/or direct bilirubin is filtered by the kidneys and is also excreted into the urine (called urine bilirubin). On the other hand, indirect bilirubin is not soluble in water. Therefore, it is also very important to evaluate the urine bilirubin in order to grasp the condition of the liver function [7].

Spectrophotometric methods with diazotized sulfanilic acid [8], 2,5-dichlorobenzene diazonium [9] and 3-nitroaniline [10] have been used for quantification of bilirubin in serum. Diazotized sulfanilic acid instantaneously reacts with direct (conjugated) bilirubin. However, it more slowly reacts with indirect (unconjugated) bilirubin. When alcohols such as ethanol [11] and/or mixed solution with caffeine/benzoate/acetate/EDTA [12] are added as accelerators, all bilirubin promptly react to produce azobilirubin. These methods give advantages of high precision, rapid color development and larger molar absorptivity by the azo compounds, and these methods are reliable for determination of total and direct bilirubin. However, they have disadvantage on interferences by other coexistent serum substances.

p-Dimethylaminobenzaldehyde (*p*-DMABA) dissolved in strong hydrochloric acid, so-called Ehrlich's aldehyde reagent, has been developed to detect bilirubin in urine and serum [7,13]. *p*-DMABA can directly react with bilirubin to form a green color product having an absorption maximum at 640 nm. Even though *p*-DMABA can react with the other coexisting constituents in urine such as urea and indican, the absorption of the colored products formed by the various substances is very small at 640 nm. The molar absorptivity of the *p*-DMABA–bilirubin complex is 28,000 L mol^{-1} cm^{-1} [7], and it is less than that of azobilirubin (74,400 L mol^{-1} cm^{-1}) [12]

An enzymatic assay utilizing bilirubin oxidase [14–19] is an alternative to detect total and direct bilirubin in serum and

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doi:10.1016/j.talanta.2011.09.048

plasma. Bilirubin oxidase can selectively oxidize bilirubin to biliverdin under conditions such as pH and temperature. Decrease in absorbance at 450 nm is proportional to bilirubin concentration in serum and plasma. This assay is satisfactory for the wide dynamic range and the excellent reproducibility. However, this method has disadvantages such as unsatisfactory stability of reagent after preparation, gives strong interference with coexisting substances in serum, and also is time consuming (incubation time up to 5 min) in the oxidizing of bilirubin. Moreover, the decrease in yellow color of bilirubin cannot be used for direct quantitative determination in urine because of the presence of other yellow substances.

Chemical oxidation methods for measuring bilirubin using acid ferric chloride reagent [20] and/or vanadic acid [21] were utilized to oxidize bilirubin to biliverdin as well as bilirubin oxidase. Vanadate oxidation method offers appropriate dynamic range of calibration with a high reproducibility, and good correlation. Furthermore, this method practically gives no interference by coexistent serum substances. In addition, this reagent is more stable, leading to no need for fresh preparation in the every determination. However, all of the diazo methods, enzymatic assay and chemical oxidation methods are batch-wise procedures. And also, these methods require a large amount consumption of reagent and sample and long time analysis per assay. In addition, these are low sample throughput and tiresome steps.

Flow injection analysis (FIA) with chemiluminescences (CL) [22–24] and/or spectrophotometric method [25] have been utilized for the automatic determination of total and direct bilirubin in serum with high sample throughput. Nevertheless, continuous baseline was basically necessary in a FI system which utilizes continuous reagent consumption with a large amount of waste generation.

The novel simultaneous injection effective mixing analysis (SIEMA) system has been reported in 2010 to demonstrate the palladium determination [26]. In the system, small amounts of sample and reagent were successively aspirated and simultaneously dispensed by means of a bidirectional syringe pump. As a result, waste generation can be reduced, and an effective mixing, rapid analysis and full automation can be established.

Matsudo et al. have developed micelle-mediate extraction for direct bilirubin in urine samples. The direct bilirubin in urine was concentrated into a small volume of surfactant-rich phase of *n*-octyl-β-D-thioglucoside (OTG), while the other coexisting constituents including urinary protein, ascorbic acid and saccharides were remained in the aqueous phase. The bilirubin in the micelle reacts with diazotized sulfanilic acid to form azobilirubin product, and the product shows an absorption maximum at 560 nm [27]. The method with pre-treatment is sensitive. However, an extraction method is based on the phase separation and the utilization of a well-micro-plate reader absorption spectrophotometer. In this study, a new and fully automated SIEMA system with three-channels and OTG is proposed for trace determination of direct bilirubin in urine.

2. Experimental

2.1. Reagents and chemicals

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

A stock standard solution of direct 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 an amber glass bottle for protection of photo-oxidation process of bilirubin and stored in −80 °C refrigerator.

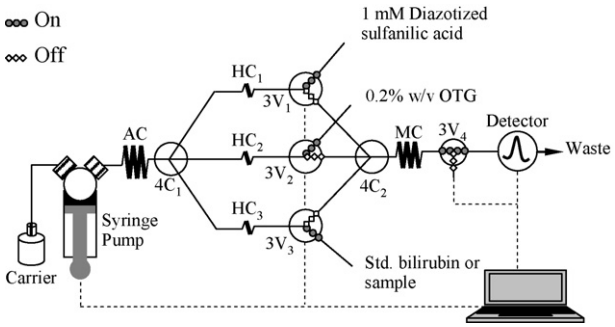

Fig. 1. The schematic diagram of the SIEMA system to determine bilirubin: AC, auxiliary coil (100 cm, 1.5 mm i.d.); $4C_1$ and $4C_2$, 4-way cross-connectors; HC_1, HC_2, and HC_3, holding coils (100 cm, 0.8 mm i.d.); $3V_1$, $3V_2$, $3V_3$, and $3V_4$, 3-way solenoid valves; MC, mixing coil (50 cm, 0.8 mm i.d.).

A stock solution of sulfanilic acid (80 mM) was prepared by dissolving 0.6962 g of sulfanilic acid (Wako Pure Chemical Co., Japan) in 0.3 M sulfuric acid to have a volume of 50 mL.

Sodium nitrite stock solution (200 mM) was prepared by dissolving 0.2774 g of sodium nitrite (Wako Pure Chemical Co., Japan) in water to be 20 mL.

The working solutions of diazotized sulfanilic acid were prepared by mixing appropriate volumes of 80 mM of sulfanilic acid solution and of 200 mM sodium nitrite solution, and then diluting to 10 mL with water. This reagent was prepared fresh daily.

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

2.2. SIEMA system

A SIEMA system for direct bilirubin determination was fabricated as shown in Fig. 1. A bidirectional syringe pump (5000 μL, CAVRO, USA) and solenoid valves (Takasogo Electric, Japan) were used to control carrier and reagents in the system. The flow lines were of Teflon tubing (0.8 mm i.d.). A visible spectrophotometer (Soma Optics, S-3250, Japan) was utilized for monitoring OTG-azobilirubin at 560 nm. The SIEMA-MPV ver. 1.4 (M&G Chematechs, Okayama, Japan) and Chromato-PRO (Runtime Instruments, Japan) were employed for automatic control and data acquisition.

2.3. Operational steps

The operational steps for standard direct bilirubin solution and/or urinary samples are listed in Table 1. All flow lines were filled with water as carrier. Diazotized sulfanilic acid, OTG and standard direct bilirubin or sample via $3V_1$, $3V_2$, and $3V_3$ were simultaneously aspirated to each holding coil (HC_1, HC_2, and HC_3) using a syringe pump. The total volume of these three solutions was 750 μL, and the volume of each reagent was approximately 250 μL. After that, the syringe pump was switched to valve in for aspiration of 4250 μL water. Then all aspirated zones were concurrently dispensed toward the detector for monitoring absorbance of azobilirubin at 560 nm.

3. Results and discussion

3.1. Detection reaction

The detection principle for direct bilirubin determination is based on the formation of direct bilirubin coupled with diazotized sulfanilic acid which is produced from sulfanilic acid with sodium nitrite [28]. Fig. 2 shows absorption spectra for diazotized

Table 1
Operational steps for determination of direct bilirubin in urinary samples.

Steps	Valve of syringe pump	V_1	V_2	V_3	V_4	Flow rate ($\mu L\,s^{-1}$)	Volume (μL)	Operations
1	Out	On	On	On	Off	100	750[a]	Simultaneous aspiration diazotized sulfanilic acid, OTG and std. bilirubin or sample to HC_1, HC_2 and HC_3
2	In	Off	Off	Off	Off	500	4250	Aspiration carrier solution to syringe
3	Out	Off	Off	Off	On	100	5000	Dispensing all aspirated zones to detector simultaneously

[a] Aspiration volume of each reagent into holding coil was approximately 250 μL (total volume was exactly 750 μL).

sulfanilic acid, direct bilirubin (5 mg L^{-1}) and azobilirubin product. Absorption maximum wavelength for direct bilirubin was presented at 450 nm and that for azobilirubin appeared at 560 nm and no absorbance from others was observed at the wavelength. The absorbance due to azobilirubin is proportional to the concentration of direct bilirubin.

3.2. Effect of diazotized sulfanilic acid concentration

The effect of concentration of diazotized sulfanilic acid solution on the sensitivity was investigated in the range of 0.1–3.0 mM. It was found that the absorbance was practically constant when the concentration of diazotized sulfanilic acid was over 1.0 mM. Therefore, 1.0 mM of diazotized sulfanilic acid was selected for further study.

3.3. Effect of OTG concentration

Matsudo et al. added OTG to extract and separate bilirubin into surfactant-rich phase. In this study, the effect of OTG was also investigated. The OTG concentration was varied in the range of 0–0.7% (w/v). As shown in Fig. 3, the absorbance without OTG was around 0.006 and it increased with increase of OTG concentration, and OTG at 0.2% (w/v) gave the larger absorbance. However, its concentration is lower than that of the critical micelle concentration (CMC: 7.5 mM at 30 °C, approximately 0.231%, w/v). It seems that OTG might slightly serve as a solubilizing agent for azobilirubin although the occurred chemical interaction is not obvious.

3.4. Effect of concurrent aspirate volume of reagents and standard/sample

To minimize the consumption of the reagent, OTG and standard/sample volumes that concurrently aspirated into each holding coil while maintaining the highest sensitivity (absorbance) were optimized. The influences of contemporary aspirate volumes of diazotized sulfanilic acid, OTG and standard/sample were studied between 300 and 900 μL of total volume. It is assumed that the solution of each line would be drawn equally (i.e. between 100 μL and 300 μL). The result is given in Fig. 4. It can be observed that the sensitivity increased with increasing aspirate volume. However, the sensitivity at aspirate volume of 750 μL (including 250 μL of 1.0 mM diazotized sulfanilic acid, 250 μL of 0.2% (w/v) OTG and 250 μL of standard/sample) was sufficient for direct bilirubin determination. Moreover, this volume provided a smooth baseline and yielded high reproducibility. Then, 750 μL of concurrent aspirate volume was chosen for the proposed method.

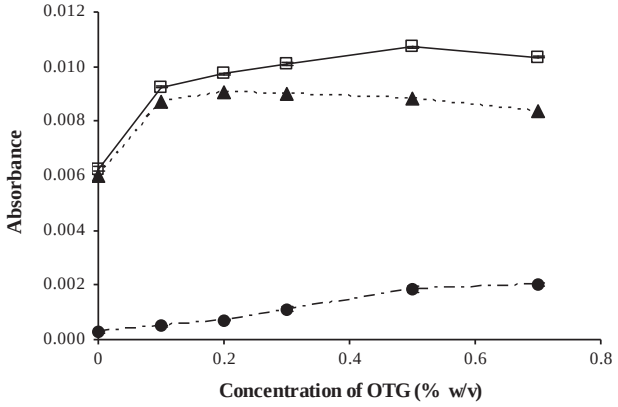

Fig. 3. Effect of OTG concentration on the 1.0 mg L^{-1} bilirubin determination; 1 mM diazotized sulfanilic acid, 750 μL aspirate volume, 50 cm mixing coil, dispensing flow rate 100 μL s^{-1}, blank signal (●), net signal (▲), and 1.0 mg L^{-1} bilirubin (□).

3.5. Effect of mixing coil length

The mixing of the reagent and analyte within the SIEMA system is very important to produce azobilirubin. The effect of mixing coil

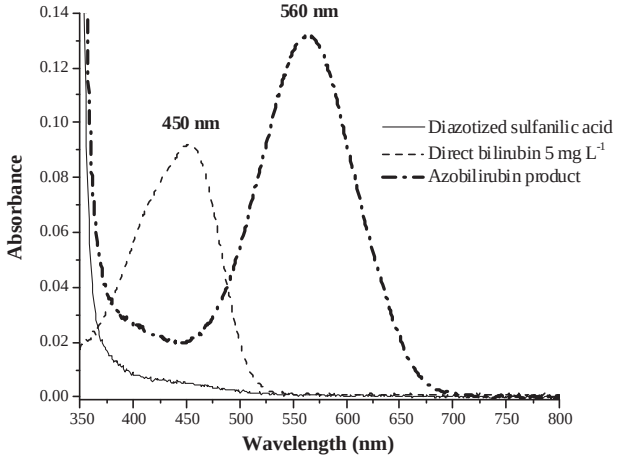

Fig. 2. Absorption spectra of diazotized sulfanilic acid, 5 mg L^{-1} direct bilirubin, and azobilirubin product by batch-wise method.

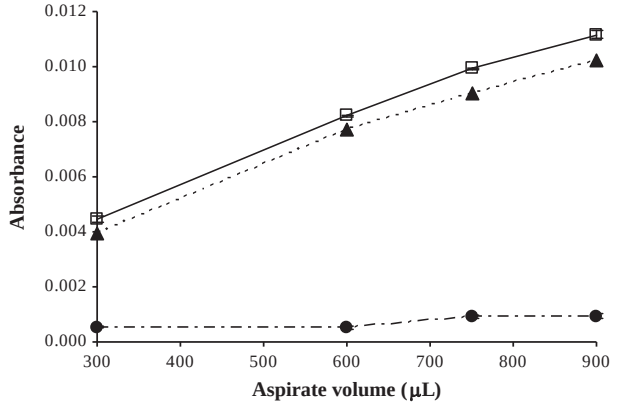

Fig. 4. Effect of simultaneous aspirate volume; 1.0 mg L^{-1} direct bilirubin, 1 mM diazotized sulfanilic acid, 0.2%(w/v) OTG, 50 cm mixing coil, dispensing flow rate 100 μL s^{-1}, blank signal (●), net signal (▲), and 1.0 mg L^{-1} bilirubin (□).

Table 2
Tolerance limits to foreign substances for 1.0 mg L^{-1} direct bilirubin determination.

Substances	Tolerance limit (mg L^{-1}) (maximum concentration causes a deviation of ±5%)
Sodium chloride	500
Potassium chloride	1000
Ammonium chloride	1000
Magnesium chloride	2000
Calcium chloride	1000
Sodium sulfate	500
Albumin	100
Ascorbic acid	100
Glucose	2000
Urea	2000
Creatinine	1000

length was also investigated at 30–150 cm. It was found that the sensitivity was increased with the mixing coil increasing until to 50 cm. After that, the sensitivity remained constant. Therefore, a mixing coil length of 50 cm was selected for rapid direct bilirubin detection.

3.6. Effect of dispensing flow rate

The dispensing flow rate concerning the sensitivity and sample throughput for bilirubin analysis was studied from 50 to 150 μL s^{-1}. The net signals were not significantly different. So, the dispensing speed at 100 μL s^{-1} was selected.

3.7. Analytical characteristics

Under the selected conditions, the calibration graph for direct bilirubin was obtained in the range 0–1.0 mg L^{-1}, with linear equation: [absorbance] = 0.00940[direct bilirubin] – 0.00017; r^2 = 0.994. A typical calibration sequence is shown in Fig. 5. The sample throughput of the proposed method was 45 h^{-1}. The relative standard deviation (RSD) for 0.5 mg L^{-1} direct bilirubin with 11 runs was 1.9%. The detection limit (LOD, 3σ) and quantitation limit (10σ) were 4.7 μg L^{-1} and 15 μg L^{-1}, respectively. The LOD with FIA/CL coupled with cloud point extraction was 1.2 μg L^{-1} and sensitivity was excellent [22], however, the LOD obtained by the proposed method was lower and more sensitive compared with other methods [23,24].

3.8. Interferences study

The effects of various foreign substances in urine on the determination of 1.0 mg L^{-1} direct bilirubin by the proposed method were investigated. The results are summarized in Table 2. In normal urine, the major remaining constituents are urea, chloride, sodium, potassium, and creatinine [29]. It was confirmed that urea up to 2000 mg L^{-1} did not interfere. Sodium chloride, potassium chloride,

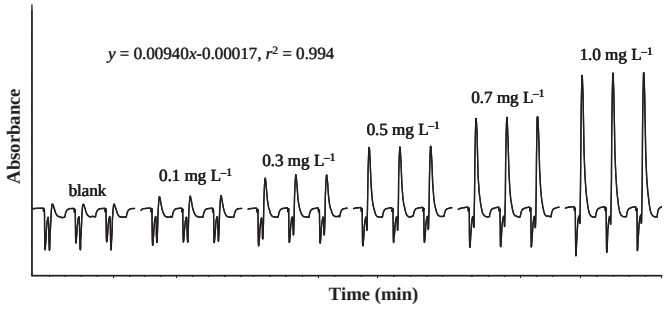

Fig. 5. FI response on calibration graph for direct bilirubin in the range of 0–1.0 mg L^{-1}.

Table 3
The concentrations of direct bilirubin in 10 healthy adults urine determined by the developed method and the batch-wise diazo method.

Samples	Batch-wise (mg L^{-1}, n = 2)	SIEMA (mg L^{-1}, n = 3)
1	0.3	0.39 ± 0.01
2	0.3	0.40 ± 0.01
3	2.8	2.68 ± 0.03
4	0.9	1.29 ± 0.04
5	0.7	0.81 ± 0.02
6	1.5	1.70 ± 0.06
7	3.8	3.81 ± 0.10
8	1.5	1.11 ± 0.04
9	1.2	1.27 ± 0.08
10	2.5	2.59 ± 0.07

n, number of experiments.

and creatinine did not interfere up to 500 mg L^{-1}, 1000 mg L^{-1}, and 1000 mg L^{-1}, respectively. Ascorbic acid usually found to be 37 mg L^{-1} in healthy subject did not interfere up to 100 mg L^{-1}.

3.9. Effect of sample dilution

In order to defeat the possible interferences from coexisting substances, it is necessary to dilute the real urine before measurement. Recovery tests of spiked bilirubin (0.5 mg L^{-1}) from urinary samples were carried out. The recovery without dilution (actually the dilution ratio was 1.01 by spiking a standard solution of direct bilirubin) was 95 ± 1.6% (n = 3). When the dilution ratio was 2, a better recovery of 100 ± 0.2% (n = 3) was obtained. Therefore, 2-fold dilution before measurement was adapted for further experiments.

To confirm the accuracy of the proposed method, other concentrations of direct bilirubin standard solutions of 0.3 and 0.7 mg L^{-1} were spiked into urine samples with 2-fold dilution. Satisfactory results (n = 3) were obtained with recoveries of 98 ± 1.1% and 100 ± 1.0% for 0.3 and 0.7 mg L^{-1} bilirubin.

3.10. Application to real samples

The proposed SIEMA method was applied to the determination of direct bilirubin in 10 urine samples taken from healthy adults. The samples were diluted at least 2 folds with water. The results obtained by the proposed method were compared with the batch-wise diazo method [8]. The results are summarized in Table 3. The correlation of direct bilirubin concentrations obtained by the proposed SIEMA method and those obtained by the batch-wise diazo method was: y = 0.944x + 0.186 with correlation coefficient (r) of 0.986. The experimental t-value between the two methods was 1.09. The t-value was less than the critical t-value (2.26) for 9 degrees of freedom at the 95% confidence level.

The determinable concentration of direct bilirubin in healthy adult urine by the proposed method and diazo methods [30,31] and p-DMABA [7] were achieved in good agreement. In a clinical laboratory, the urinary bilirubin level was an important marker for diagnosis on liver damage such as jaundice, hobnailed liver and hepatitis. Usually it is reported as a negative or positive result and the upper normal level urine bilirubin may be set at 1 mg L^{-1} [32]. The proposed SIEMA is an alternative and useful method to a rapid, simple, precise and fully automated determination of direct bilirubin in urine.

4. Conclusion

A simple, fully automated operation, sensitive and accurate simultaneous injection effective mixing analysis system using spectrophotometry for direct bilirubin was developed. The reaction is based on the formation of direct bilirubin coupled with diazotized

sulfanilic acid in the presence of OTG. The proposed method was successfully applied to determine small amounts of direct bilirubin in urinary samples. The proposed new SIEMA system would be useful for liver function test in the clinical laboratory.

Acknowledgements

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. and T.S. gratefully acknowledge the financial support of this study by Grants-in-Aid for Scientific Research Nos. 22550085 (to N.T.) and 21550093 (to T.S.) from Japan Society of the Promotion of Science.

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