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Tandem measurements of iron and creatinine by cross injection analysis with application to urine from thalassemic patients ☆

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ABSTRACT

This work presents development of a method for the dual determination of Fe(III) and creatinine using cross injection analysis (CIA). Two CIA platforms connected in series accommodated sample and reagents plugs aspirated via *y*-direction channels while water was pumped through the *x*-direction channel toward a flow-through cell of a diode array UV–vis. detector. Iron was detected from the colorimetric reaction between Fe(II) and 2-(5-bromo-2-pyridylazo)-5-(*N*-propyl-*N*-(3-sulfopropyl) amino) aniline (5-Br-PSAA), with prior reduction of Fe(III) to Fe(II) by ascorbic acid. The Jaffe's reaction was employed for the detection of creatinine. Under the optimal conditions, good linearity ranges were achieved for iron in the range 0.5 to 7 mg L^{-1} and creatinine in the range 50 to 800 mg L^{-1}. The CIA system was applied to spot urine samples from thalassemic patients undergoing iron chelation therapy, and was successfully validated with ICP-OES and batchwise Jaffe's method. Normalization of urinary iron excretion with creatinine is useful for correcting the iron concentration between urine samples due to variation of the collected urine volume.

1. Introduction

Cross injection analysis (CIA) is a recent generation of flow injection analysis (FIA) and sequential injection analysis (SIA) [1,2]. The concept of CIA is based on the use of a small acrylic platform with dimension of 5 cm × 3 cm × 1.5 cm ($x \times y \times z$). The acrylic platform is drilled to give a very simple system of cylindrical channels with two or more *y*-channels running perpendicularly to the *x*-channel. The i.d. of the channels can vary from 1 to 3 mm depending upon the amount of the liquids we wish to introduce into the flow system. The *y*-channel is used as both the inlet and the outlet ports for either sample or reagents. The *x*-channel is the main or analytical flow path that connects to the detection flow cell. The inlet and outlet of every channel are connected to pump tubes that are partially wound on the rollers of peristaltic pumps.

CIA is in a way similar to SIA in term of insertion of liquid zones into the analytical flow path (*x*-channel) before detection. The difference is that zone insertion of both sample and reagents in CIA are carried out simultaneously not sequentially as in SIA. The technique of cross-flow promotes the mixing between the samples and reagents, leading to improvement in the sensitivity. Liquid handling in CIA requires only peristaltic pumps with an electronic control board for starting/stopping the pumps. CIA is somewhat similar to the technique of multicommutation [3]. In the multi-commutation flow system, solenoid valves are employed and set at the close-position to maintain constant pressure of the liquids inside the tubing. In CIA, the pressure during the stopped-flow mode of the peristaltic pumps is also constant; adequate to hold the liquid inside the tubing without the need of solenoid valves. The programming steps for the electronic control board are also simple and short. The first application of CIA was demonstrated for the determination of Fe

☆This paper is dedicated to Prof. Kate Grudpan on the anniversary of his 60th Birthday.

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(II) and Fe(III) in multi-vitamin tablets [4]. The detection was based on use of 1,10-ortho-phenanthroline as the complexing agent to form red-colored Fe(II) product.

This work extends application of the CIA technique to determine iron excreted in urines of thalassemic patients. Normally iron is not found in urine. However thalassemic patients undergoing an iron chelating therapy will excrete iron in urine. Genetic defect in thalassemia results in abnormal hemoglobin synthesis and subsequent shortened life-span of the red blood cell, leading to excess iron accumulation within tissues. Iron chelation is required to remove the excess iron which leads to tissue damage [5]. Monitoring of the level of iron in a patient's urine indicates the efficacy of the therapy and also the iron-overload status [6–10].

Deferiprone (1,2 dimethyl-3-hydroxypyrid-4-one, also known as L1) is an orally active iron chelator that is commonly used as life-saving drug for thalassemic patients in several countries, including Thailand. In urine samples of thalassemic patients undergoing treatment with deferiprone, the excreted iron is present in the form of tris-deferiprone iron(III) complex. There are only a few reports for quantitative analysis of iron excreted in urine as iron-drug complexes. Leflon and Plaquet presented an ICP-OES method for determination of iron excreted after the treatment with the chelator deferoxamine [7]. Lundvall and Weinfeld presented a colorimetric method for the determination of iron in urine after a chelator treatment using deferoxamine [6]. To our knowledge, there are few of reports of method for determining deferiprone iron in urine of thalassemic patients [8–10]. In these articles, deferiprone iron was determined based on colorimetric detection using ferrozine [8] or bathophenanthroline disulfonic acid (batho-phen) [9,10] as the chromogenic agent. In the batho-phen colorimetric method, nitrilotriacetic acid (NTA) is added into urine to scavenge Fe(III) from the Fe(III)–deferiprone complex [9,10].

$$\mathrm{Fe(III)-(L1)_3} \xrightarrow{\mathrm{NTA(excess)}} \mathrm{Fe(III)-NTA+3(L1)} \quad (1)$$

$$\mathrm{Fe(III)-NTA} \xrightarrow{\mathrm{TGA(excess)}} \mathrm{Fe(II)-NTA} \quad (2)$$

$$\mathrm{Fe(II)-NTA} \xrightarrow{\mathrm{batho-phen(excess)}} \mathrm{Fe(II)-(batho-phen)_3+NTA} \quad (3)$$

In this work, the CIA technique with spectrometric detection was utilized in the development of a new method for the tandem measurements of deferiprone-bound iron and creatinine in human urine. The batch method [9,10] was modified by employing 2-(5-bromo-2-pyridylazo)-5-(*N*-propyl-*N*-(3-sulfopropyl)amino) aniline (5-Br-PSAA) as the complexing agent and ascorbic acid as the reducing agent for better sensitivity. Creatinine was detected using the Jaffé method [11–15], in which creatinine reacted with alkaline picrate to give a colored product. The tandem measurements of iron and creatinine in urine by CIA provide a simple and convenient method for spot urine samples. The measurement of creatinine in the urine is used to correct for the variation in the volume of the urine collected.

Two acrylic platforms connected in series were used in the CIA flow system to produce two plugs of reaction zones, one containing Fe (II)–5-Br-PSAA complex and the other the product of the Jaffé reaction, flowing in the *x*-channels. These separate reaction zones were successively delivered by the carrier (*x*-directional flow) into the flow-cell, giving rise to a peak of iron followed by the creatinine peak.

2. Experimental

2.1. Preparation of reagents and samples

All chemicals used in this work were analytical-reagent grade. Deionized-distilled water was used throughout all the experiments. Acetate buffer (0.5 mol L^{-1}) was employed to maintain the pH of solutions.

An iron standard solution containing 1000 mg Fe(III) L^{-1} in 0.5 M HNO_3 for atomic absorption spectrophotometry (AAS) (Merck, Germany), was used as the primary standard for Fe(III). A solution of pure deferiprone drug (1000 mg deferiprone L^{-1}) was prepared by dissolving 0.05 g of deferiprone powder (Aldrich, Germany) in 50 mL of water. The stock of Fe(III)–drug complex (solution A), containing Fe(III) at 100 mg Fe(III) L^{-1}, was prepared by mixing 5.00 mL of the standard Fe(III) solution for AAS (1000 mg Fe(III) L^{-1}) with 40.0 mL of the drug solution (1000 mg deferiprone L^{-1}), and made up with water to 50.0 mL in a volumetric flask. The ratio of Fe (III) to drug is 1:3.2. The ratio was set to slightly greater than 1:3 to ensure the complete formation of the Fe(III)–$(L1)_3$ complex.

Stock creatinine at 5000 mg creatinine L^{-1} (solution *B*) was made by dissolving an accurate weight of approximately 0.5 g of creatinine anhydrous (Sigma, USA) in 100.0 mL of water. A 25 mL nitrilotriacetic acid (H_3NTA) solution (0.2 mol L^{-1}) was prepared by dissolving 0.95 g of H_3NTA powder (Fluka, Switzerland) in a small amount of water. Sodium hydroxide solution (6 mol L^{-1}) was used for aiding the dissolution of the solid and for adjusting the final solution to pH 7. This solution was used as the chelating reagent to remove Fe(III) from deferiprone drug before reduction and formation of the colored Fe(II)-complex.

The standard working solutions used for calibration in CIA, was a mixture of aliquots of Fe(III)–drug (solution *A*), creatinine (solution *B*), NTA stock solution and buffer. The standard solutions were prepared to have Fe(III) and creatinine in the concentration range 0.5 to 7 mg Fe (III) L^{-1} and 50 to 800 mg creatinine L^{-1}, respectively. All working solutions were prepared daily in 10-mL volumetric flasks, with addition of 450 μL NTA (0.2 mol L^{-1}) and 4.0 mL of acetate buffer (0.5 mol L^{-1}) into each flask, and made up to volume with water (final pH 4.4).

In the analysis of iron, the 5-Br-PSAA reagent (0.3 mmol L^{-1}), designated as R1 in the CIA system in Fig. 1, was made by appropriate dilution with water from the stock reagent (2 mmol L^{-1}) prepared from solid powder of the 2-(5-bromo-2-pyridylazo)-5-(*N*-propyl-*N*-(3-sulfopropyl)amino) aniline (Dojindo, Japan). The solution of ascorbic acid (25 mmol L^{-1}), designated as R2 in Fig. 1, was prepared by dissolving 0.11 g of L-ascorbic acid (Fisher Scientific, UK) in 10 mL of the acetate buffer and was further diluted with water to 25 mL (final pH 4.4). For the analysis of creatinine, an aqueous saturated solution of picric acid (ca. 52 mmol L^{-1}) was first prepared using picric acid powder (Merck, Germany). The alkaline picrate solution, designated as R3 in Fig. 1 (25 mmol L^{-1} in 0.4 mol L^{-1} NaOH) was prepared by mixing 12 mL of the saturated solution of picric acid with 4 mL of 2.5 mol L^{-1} NaOH solution and 9 mL water.

Urine samples in this work were spot urines, collected from thalassemia patients with intermediate and severe iron overload. After filtration through a 0.45-μm cellulose membrane filter, each sample was pipetted (1.00 or 2.00 mL) into a 10-mL volumetric flask, followed by additions of 450 μL NTA (0.2 mol L^{-1}) and 4.0 mL acetate buffer (0.5 mol L^{-1}). The mixture was made up to 10.0 mL with water. The sample was left to stand for at least 30 min to allow for NTA to completely displace the L1 drug from Fe (III) [9,16] prior to injection into the CIA platforms via the no .1 and no .4 *y*-channels (designated as S in Fig. 1).

2.2. The CIA system and its operation

Fig. 1 is a schematic of the CIA flow-manifold. The complete system with details of connections with pump tubes and peristaltic pumps are given in the Supplementary material Fig. 1S. The CIA system comprised two separate CIA platforms (Fig. 2S), namely the 'iron platform' and the 'creatinine platform'. These platforms were made of acrylic material similar to that described in the previous

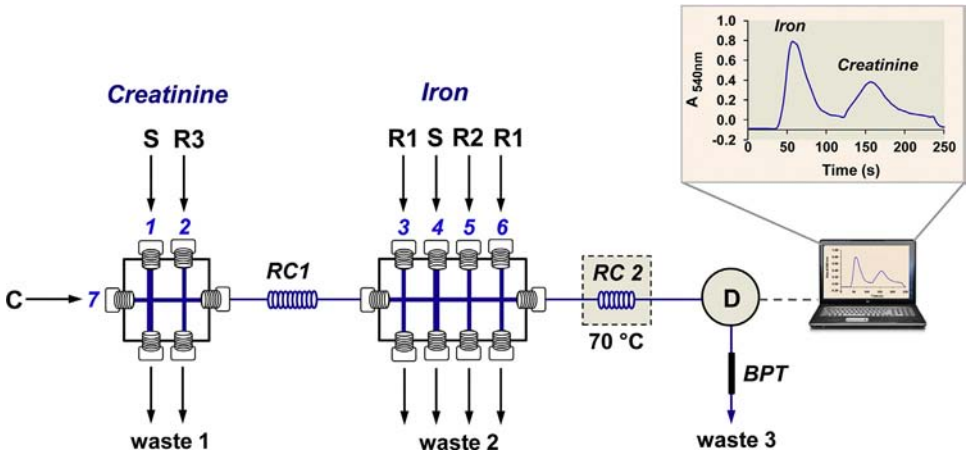

Fig. 1. The simplified CIA manifold for dual determination of iron and creatinine. C: Water, S: Sample, R_1: 0.025 mol L^{-1} picric acid in 0.4 mol L^{-1} NaOH, R_2: 3.0 mmol L^{-1} 5-Br-PSAA and R_3: 25 mmol L^{-1} in 0.4 mol L^{-1} acetate buffer pH 4.4, RC1 and RC2: Reaction coil with 200 and 100 cm, respectively and D: Diode array detector (Iron: λ=558 nm, creatinine: λ=520 nm).

Table 1
Operation steps optimized for the CIA system (Fig. 1S) for tandem measurements of iron and creatinine in human urine.

Step	Process	P1	P2	P3	Duration time (s)
1	Loading of sample, reagents and carrier	On	On	On	10
2	Zone transporting to reaction coils	Off	On	Off	10
3	Zone stopping in reaction coils	Off	Off	Off	120
4	Zone flushing for detection	Off	On	Off	215

P1, P3: peristaltic pumps for sample and reagents; P2: peristaltic pump for carrier.

work [4]. The platforms were used for individual introduction and mixing of the sample and reagents for iron and creatinine, respectively. PTFE tubings with 1.0 mm i.d. (Cole Parmer, USA) were employed for the connecting tubings and reaction coils (RC1 and RC2). An Ismatec pump (model IS7610, Switzerland, designated as P1 (Fig. 1S), was used for the simultaneously filling and of withdrawing the sample/standard (S) in the *y*-channels (channels 1 and 4) for both CIA platforms. A second Ismatec pump of the same model (P2) was employed for driving the water carrier (C) through the *x*-channel (channel 7) of the system. A third Ismatec peristaltic pump (model ISM843, Switzerland, designated as P3 in Fig. 1S) with capacity for accommodating eight tubings, was used for simultaneously filling and withdrawing of the three reagents (R1, R2 and R3), in the *y*-channels (channel 2 in the 'creatinine platform' and channels 3, 5 and 6 in the 'iron platform').

The flow line of the CIA platforms in the *x*-direction is finally connected to the flow cell of the UV–vis. spectrophotometer D (Agilent™ UV–vis. diode array spectrophotometer, model 8453, Germany), equipped with a Perkin Elmer™ 10-mm path-length flow-through cell (18 μL). Monitoring of the reaction products was at 558 or 540 nm for iron, and at 520 or 540 nm for creatinine. Fisher Scientific™ thermostated bath (Isotemp 205, USA) was used for heating the reaction coil RC2 to improve the sensitivity of iron analysis.

An in-house electronic control board was used to start/stop the peristaltic pumps P1, P2 and P3 using software written in Visual Basic 6.0. The steps of operation for the optimized condition are given in Table 1. In step 1, cross-flow was applied for both platforms to load the sample and reagents into the analytical path via all of the *y*-channels. At the same time, the carrier was also pumped through the *x*-channel of both platforms. Simultaneous flow in both *x*- and *y*-directions promoted turbulent mixing of the sample and reagents within the crossing zones of the platforms. In step 2 and step 3, the reaction zones of iron and creatinine were propelled into reaction coils RC1 and RC2, respectively, and the flow then stopped for 20 s to allow progression of the reactions. Finally, the reacted zones were pushed into the detection cell in step 4.

3. Results and discussion

3.1. Selections of chromogenic and reducing reagents for iron

Bathophenanthroline disulfonate (batho-phen) has often been employed as a chromogenic reagent for determination of iron in biological samples [9–10,16–18]. Sakai and co-workers have shown that 5-Br-PSAA is also an effective chromogenic reagent for Fe(II) and demonstrated its application in the determination of serum iron [19,20]. Batch experiments were carried out to compare the performance of batho-phen and 5-Br-PSAA. In these experiments, thioglycolic acid (TGA) was used as the reducing agent at pH 7 for Fe(II)–batho-phen, using a HEPES buffer, and at pH 4.2 for Fe(II)–5-Br-PSAA, using acetate buffer.

The maximum absorption wavelength (λ_{max}) in our condition for the Fe(II)–batho-phen complex was observed at 537 nm. The λ_{max} for Fe(II)–5-Br-PSAA was observed at 558 nm, which agreed with the previous report [21]. The absorbance measured at a fixed time of 3 min, was used for the calibration plots. Linear calibrations were obtained for both reagents for 0.28 to 0.84 mg Fe(II) L^{-1}. Results showed that the 5-Br-PSAA reagent is more sensitive than batho-phen [Fe(II)–batho-phen: $A_{537}=0.0230\times(\text{mg Fe(II) L}^{-1})+0.0121$, $r^2=0.998$ and Fe(II)–5-Br-PSAA: $A_{558}=0.0535\times(\text{mg Fe(II) L}^{-1})+0.0646$, $r^2=0.998$]. Therefore, 5-Br-PSAA was selected for this work as the chromogenic reagent for iron with detection at 558 nm.

Two reducing agents were compared, namely thioglycolic acid and ascorbic acid. Experiments were carried out using Fe(III) bound to the NTA ligand. Solution of Fe(III)–NTA complex was pre-mixed with either thioglycolic acid or ascorbic acid before adding 5-Br-PSAA. It was observed that the kinetics of formation of Fe(II)–5-Br-PSAA was much faster with ascorbic acid than thioglycolic acid (Fig. 2). Ascorbic acid was therefore chosen as the reducing agent in this work.

3.2. Optimization of the CIA system

3.2.1. System design and detection sequence

The CIA system (Figs. 1 or 1S) consists of two acrylic platforms placed in tandem for the formation of separate reaction zones for the analysis of iron and creatinine. The *y*-channels of the platforms were used to load the sample and reagents. The *x*-channels of the

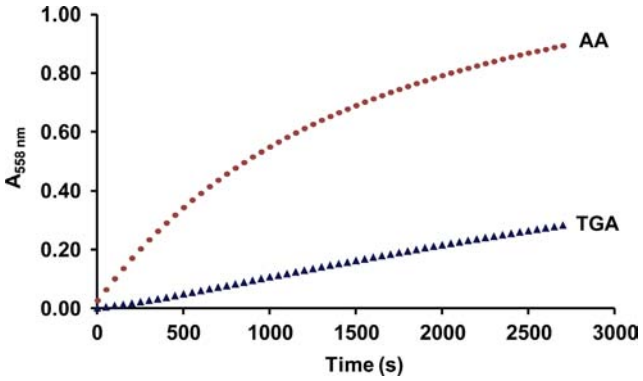

Fig. 2. Kinetics study of the color developing reaction between Fe(II) and 5-Br-PSAA using either ascorbic acid (AA) or thioglycolic acid (TGA) as reducing agent for prior reduction of Fe(III) to Fe(II).

platforms are connected to reaction coils RC1 and RC2 to form an analytical flow-path to the detection cell. A diode-array spectrophotometer was used for simultaneous measurements of absorbances at two different wavelengths. The wavelengths selected for iron and creatinine were 558 nm (Fe(II)–5-Br-PSAA, [19,21,22]) and 520 nm (creatinine–picrate complex, [23]), respectively. In order to minimize dilution of the iron reaction zone, the CIA platform and reaction coil (RC2) were placed closest to the detector. Since the level of creatinine is much greater than iron, the position of creatinine platform does not compromise its sensitivity. In our configuration, the reaction zone of iron was first detected followed by the reaction zone of creatinine. Thus, we obtain two consecutive signals for iron and creatinine, respectively, as shown in the inset of Fig. 1.

3.2.2. Zone sequence

The sequence of the reagent and sample liquid zones was investigated using platforms with 2-mm i.d. channels. The results, as shown in Fig. 3a, showed that selection of the zone sequence markedly affected the sensitivities of both iron and creatinine analysis. For iron, the sensitivity was significantly increased when the zones of iron and the buffered ascorbic acid (AA) were sandwiched between the zones of 5-Br-PSAA (sequence a1). There may be insufficient amount of 5-Br-PSAA using sequence a2 as compared to sequence a1, since in sequence a2 there was only one inlet port for introducing the reagent. Thus, sequence a1 was selected for iron.

For creatinine, we compared two sequences as shown in Fig. 3b, and found that sequence b1 gave a better sensitivity than sequence b2. In sequence b1 alkaline picrate was placed as the front zone during the cross-flow and consequents, there was only slight loss, via y-channel no. 2, of the picrate reagent to waste 1 (Fig. 1). In sequence b2, the front zone was the analyte zone whereas the rear zone was the picrate zone. Some of picrate reagent in sequence b2 was lost to waste 1 through both *y*-channels, i.e., channels no. 1 and no. 2, during the cross-flow.

3.2.3. Channel diameter

The inner diameter of the *y*-channel used for the sample introduction was compared between sizes 2 mm and 3 mm, respectively. The inner diameter of the other channels was fixed at 2 mm. The calibration graphs for iron and creatinine showed that sensitivities were improved for the larger diameter of the *y*-channel for the sample. The slope of the calibration line for iron slightly increased (1.1 ×) when increasing the diameter from 2 to 3 mm. However the calibration slope was considerably increased (1.4 ×) for creatinine. In further development study, the *y*-channels for the sample in both platforms were kept at 3 mm i.d.

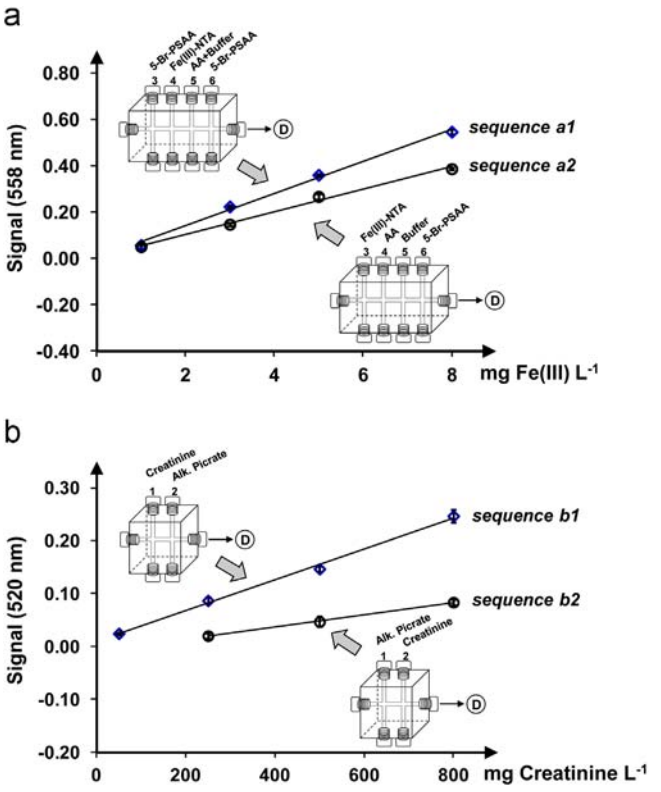

Fig. 3. Effect of zone sequence on the sensitivity for analyses of (a) iron and (b) creatinine. Note: see Table 1S for details of the reagents.

3.2.4. Reaction temperature for iron determination

In iron chelation therapy with deferiprone, the concentrations of iron in patients' urine are considerably much lower than creatinine. The levels of excreted iron found in patients' urine can be as low as 0.7 mg Fe(III) L^{-1} [9], whereas creatinine normally vary from 620 to 1880 mg creatinine L^{-1} [13]. We therefore focused on improving the sensitivity of the iron determination. Since the rate of reaction between Fe(II) and 5-Br-PSAA increases with temperature [19,20,22], the reaction coil, RC2, was immersed in a thermostated bath and the bath temperature varied from 30 °C to 70 °C. As expected, the sensitivity increased as we increased the temperature. In this work, the bath temperature of 70 °C was selected. At this temperature, it was also necessary to place a stainless steel coil (i.d.: 0.5 mm, length: 200 mm) at the outlet end of the detection cell to maintain back pressure to inhibit formation of air bubbles in the flow line.

3.2.5. Flow rate and stopped-flow period

The effect of the *y*-channel flow rate was studied for flow rate from 0.6 to 1.2 mL min^{-1}. The flow rate of the *x*-channel was fixed at 1 mL min^{-1}. It was found that the flow rate has a larger effect for creatinine than for iron. The sensitivity increased with increasing flow rates of the creatinine platform. It was decided to set the flow rate for all flow channels at 1.0 mL min^{-1} for every operational step.

A cross-flow of 1.0 mL min^{-1} for both *x*- and *y*-channels was first applied for 10 s to introduce sample and reagents into the platforms via *y*-channels. The *x*-directional flow was then continued for a further 10 s to transport the reaction zones of iron and creatinine into the reaction coils RC2 and RC1, respectively. The flow was stopped for a fixed period to allow reaction to progress zone in the reaction coil. The time for stopped-flow was optimized for iron by selecting 1-, 2- or 3-min periods, respectively (data not shown).

As reported by Sakai's group [19,20,22] that the reaction between Fe(II) and 5-Br-PSAA was accelerated by temperature, we also observed that increasing the temperature extensively enhanced the sensitivity for iron. The slopes of the calibration lines for the three stopped-flow modes were all greater than the slope for normal continuous flow mode. The slope for 3-min stopped-flow period was ca. 1.5 times greater than that for 1-min stopped-time. However, we selected 2 min as the stopping period. At 2 min, the sensitivity for iron was sufficient and the analysis time was acceptable. Since we decided to work with a 2-min stopped-time for iron, the analysis of creatinine must therefore be operated with the same condition. The 2-min stopped-flow period also improved the sensitivity of creatinine analysis.

3.2.6. Reagent condition

In the determination of iron (Fig. 1), the concentration of 5-Br-PSAA (R1) was first optimized. Although the slope of the calibration line increased approximately 2 fold when the concentration of 5-Br-PSAA was increased from 0.2 to 0.5 mmol L^{-1}, the blank signal, however increased three fold. In view of the cost of the reagent and as a compromise between sensitivity and baseline signal, the concentration of 5-Br-PSAA reagent was set at 0.3 mmol L^{-1}. Ascorbic acid (R2) in the concentration range 10 to 100 mmol L^{-1} did not have significant effect on the signal for the 5 mg Fe(III)–NTA L^{-1} sample nor for a blank sample. We chose 25 mmol L^{-1} as the optimal concentration for ascorbic acid. The pH of R2 was also varied from pH 3.7 to 4.6, using acetate as the buffer. The pH 4.4 gave the highest signal for repetitive injections of a 5 mg Fe(III)–NTA L^{-1} sample. We therefore selected pH 4.4 for the reducing agent R2. Acetate buffer at pH 4.4 was also used as solvent for the Fe(III)–NTA standards and for diluting the urine samples.

For the creatinine analysis, the concentrations of picric acid and sodium hydroxide were examined. The sensitivity increased as we increased the picric concentration from 0.01 to 0.04 mol L^{-1}. Increasing the concentration of sodium hydroxide from 0.05 to 0.4 mol L^{-1} also improved the sensitivity for creatinine. Since the sensitivity for creatinine was not critical, the concentration of picric acid in the mid-range at 0.025 mol L^{-1} was selected. However 0.4 mol L^{-1} sodium hydroxide was selected for preparing the alkaline picrate to minimize decrease in the basicity on mixing with the urine sample which is in pH 4.4 acetate buffer.

3.2.7. Single-wavelength detection

Initially the wavelengths selected for absorbance measurement was the optimal wavelengths namely 558 nm for Fe(II)–5-Br-PSAA complex and 520 nm for the creatinine–picrate product, respectively. A diode-array spectrophotometer was thus employed for the consecutive recording. However, the absorbance bands of the two analytes have overlapping region. Therefore, it may be possible to select a common wavelength and use an alternative and low-cost detector, if the sensitivity is found to be sufficient.

Table 2
List of calibration equations at different monitored wavelengths (λ) for Fe(III)–NTA and creatinine determination.

λ (nm)	Calibration equation of Fe(III)–NTA[a]	r^2	Calibration equation of creatinine[b]	r^2
520	$y=(2.70\times10^{-2})\,x+(0.44\times10^{-2})$	0.996	$y'=(8.58\times10^{-4})\,x'+(1.18\times10^{-2})$	0.999
530	$y=(4.19\times10^{-2})\,x-(0.02\times10^{-2})$	1.000	$y'=(6.00\times10^{-4})\,x'+(4.72\times10^{-2})$	0.999
540	$y=(5.76\times10^{-2})\,x-(0.19\times10^{-2})$	0.999	$y'=(3.56\times10^{-4})\,x'+(4.65\times10^{-2})$	0.999
550	$y=(7.86\times10^{-2})\,x-(0.39\times10^{-2})$	0.999	$y'=(1.83\times10^{-4})\,x'-(1.05\times10^{-2})$	0.995
558	$y=(8.72\times10^{-2})\,x-(0.26\times10^{-2})$	0.999	$y'=(0.90\times10^{-4})\,x'-(1.14\times10^{-2})$	0.987

[a] The linear range for Fe(III)–NTA was 1.0–5.0 mg L^{-1} Fe(III)–NTA. y is the absorbance difference ($A-A_0$) and x is the concentration (mg L^{-1}).
[b] The linear range for creatinine was 150–800 mg L^{-1} creatinine. y' is the absorbance difference ($A-A_0$) and x'' is the concentration (mg L^{-1}).

Table 3
Analytical features of the CIA system for tandem measurements of iron and creatinine in patients' urine treated with deferiprone.

Feature	Iron(III)	Creatinine
1. Calibration equation	$A\text{-}A_{0\ (558nm)} = ((7.71\pm0.27)\times10^{-2})\,[Fe(III)] + ((0.43\pm1.12)\times10^{-2})$, $r^2 = 0.996$	$A\text{-}A_{0\ (520nm)} = ((1.47\pm0.02)\times10^{-3})\,[Cre] + ((1.76\pm0.79)\times10^{-2})$, $r^2 = 0.999$
	$A\text{-}A_{0\ (540nm)} = ((5.65\pm0.15)\times10^{-2})\,[Fe(III)] + ((0.44\pm0.63)\times10^{-2})$, $r^2 = 0.998$	$A\text{-}A_{0\ (540nm)} = ((0.60\pm0.003)\times10^{-3})\,[Cre] + ((4.05\pm1.23)\times10^{-3})$, $r^2 = 0.999$
2. Working range (mg L^{-1})	0.5 – 7 (558 nm) 1 – 7 (540 nm)	50 – 800 (520, 540 nm)
3. Limit of quantitation (LOQ), (y_B+3S_B, mg L^{-1})	**0.39** *(558 nm)* **0.80** *(540 nm)*	**20** *(520 nm)* **29.5** *(540 nm)*
4. Precision (%RSD)	**3.0** *(5 mg Fe(III) L^{-1}, 558 nm)* **4.7** *(5 mg Fe(III) L^{-1}, 540 nm)*	**4.8** *(250 mg Creatinine L^{-1}, 520 nm)* **5.5** *(250 mg Creatinine L^{-1}, 540 nm)*
5. Sample throughput (samples/h)	← **10** →	
6. % Recovery (n=5)	**86 - 105** *(558 nm)* **85 - 110** *(540 nm)*	**88 - 109** *(558 nm)* **83 - 111** *(540 nm)*
7. Sample consumption/ analysis (μL)	**167**	**167**
8. Reagent consumption/ analysis (μL)	**334** (5-Br-PSAA), **167** (ascorbic acid in buffer)	**167**

y_B; Blank signal, S_B; standard deviation of the blank.

The data from the diode-array detector recorded at wavelength range 400–900 nm were thus re-analyzed and calibration curves constructed using single wavelengths. Table 2 shows that linear calibration lines were obtained for all the wavelengths selected. The optimal single wavelength was 540 nm, since at wavelength greater than 540 nm the sensitivity for creatinine is too small. Wavelengths less than 540 nm are not sensitive enough to detect the low concentration levels of iron in urine samples.

3.3. Analytical features and validation

The analytical performance of the developed system is shown in Table 3. The CIA system gave the highest sensitivity with linear calibration when the absorbances of iron and creatinine reaction zones were measured at 558 nm and 520 nm, respectively. However, the absorbances of these reaction zones can also be monitored using the single wavelength at 540 nm. The linear working ranges for 540 nm cover the levels of both iron and creatinine in the urines of thalassemic patients with acceptable LOQs. Good precision was obtained for all selected wavelengths (RSD from 3.0 to 5.5%). Table 3 shows that the sample throughput of the CIA system is acceptable considering that the system gives the results of two analytes within 6 min. Data also showed that the system provided good recoveries in urine samples. The consumption of reagents is satisfactorily low, which is particularly important when employing a relatively expensive reagent like 5-Br-PSAA. In our system, the cost of 5-Br-PSAA per analysis of iron is approximately 0.07 USD.

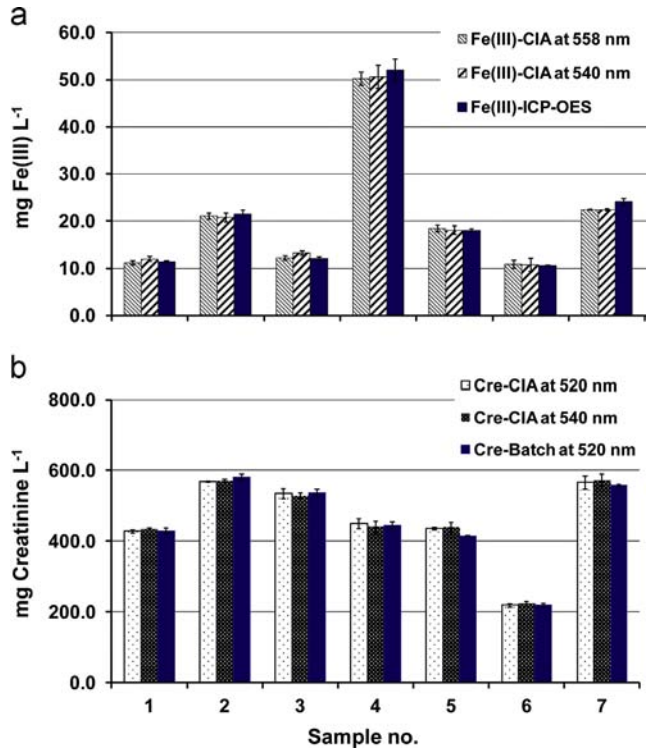

Fig. 4. Comparison of the results from CIA with the results from the validating methods, (a) iron determination and (b) creatinine determination. Note: The ICP-OES conditions for Iron(III)) are as follow: nebulizer gas flow rate; 1.0 ml min^{-1}, coolant gas flow rate; 12 ml min^{-1}, auxiliary gas flow rate; 1.0 ml min^{-1} and emission line (nm) monitored for iron; 238.204, 259.940, 239.562. The batch Jaffe's method (for creatinine) was modified from [11–15].

Table 4
The ratio of the concentration of iron to creatinine of the spot urine samples of seven patients.

Sample no.	mg Fe(III)/mg Creatinine ± SD	
	558 nm	540 nm
1	0.0261 ± 0.0012	0.0277 ± 0.0013
2	0.0371 ± 0.0012	0.0365 ± 0.0016
3	0.0228 ± 0.0009	0.0252 ± 0.0009
4	0.112 ± 0.0031	0.115 ± 0.0055
5	0.0424 ± 0.0017	0.0412 ± 0.0022
6	0.0498 ± 0.0042	0.0484 ± 0.0064
7	0.0397 ± 0.0001	0.0394 ± 0.0003

As shown in Fig. 4, the CIA method was compared with ICP-OES for iron (Fig. 4a) and with the Jaffe's batch method for creatinine determination (Fig. 4b). According to paired *t*-test, no significant different was found between the results of our method and the ICP-OES method at 95% confidence limit ($t_{observed} = -0.54$ (558 nm) and 0.32 (540 nm), $t_{critical} = 2.57$). For creatinine, the results from our method are also not significantly different to the batch method at 95% confidence limit ($t_{observed} = 0.58$ (520-nm) and −1.11 (540 nm), $t_{critical} = 2.57$).

Table 4 shows the data for the ratio of the concentration of iron to creatinine of the urine samples collected as spot urine from seven patients undergoing iron chelating therapy with deferiprone. These results can be used to monitor the progress of the chelation treatment of such patients.

4. Conclusions

The recent approach to flow injection technique, the so called 'CIA' [4], was exploited in development of a new and efficient method for monitoring the efficacy of the iron chelator deferiprone in the treatment of thalassemia. The treatment results in excretion of urinary iron in the form of Fe(III)–deferiprone complex. In former work [9,10], urine collected over 24 h was employed for the determination. In this work, the CIA system provides simultaneously quantitation of both excreted iron and creatinine. The ratio of concentrations of excreted iron and creatinine can be used to evaluate the efficacy of the chelating therapy using spot urine sample. This system may be developed as a potential technique for point of care service.

We proposed the use of 5-Br-PSAA as color forming reagent for Fe(II), which was found to give a significantly better sensitivity than bathophenanthroline disulfonate. The concentration of reagents and zone sequences were optimized for the iron determination. Nitrilotriacetic acid (NTA) chelator was employed as the scavenging agent to form Fe(III)–NTA complex from the Fe(III)–deferiprone complex in urine before the CIA analysis. Within the CIA platform for iron, Fe(III) as the NTA complex was reduced to Fe (II) which then form the reddish–purple complex of Fe(II)–5-Br-PSAA for spectrometric monitoring after a fixed-time interval. At the same time, creatinine in the sample undergoes reaction with alkaline picrate on the other CIA platform. The peak signals from the reaction zones of Fe(II)–5-Br-PSAA and of creatinine–picrate product are used to determine the concentration iron and creatinine in the urine sample. Detection at two different wavelengths for the zones of iron (558 nm) and creatinine (520 nm) gives the optimum condition using a diode-array detector. However, single wavelength detection at 540 nm was found to give an acceptable sensitivity. Hence a single wavelength can be employed.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.090.

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