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Portable microfluidic system for determination of urinary creatinine

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ABSTRACT

A simple, low cost and portable microfluidic system based on a two-point alkaline picrate kinetic reaction has been developed for the determination of urinary creatinine. The creatinine reacts with picric acid under alkaline conditions, forming an orange-red colour, which is monitored on PDMS microchip using a portable miniature fibre optic spectrometer at 510 nm. A linear range was displayed from 0 to 40 mg L^{-1} creatinine (r^2 = 0.997) with a detection limit of 3.3 mg L^{-1} (S/N = 3). On-chip absorbance signals are reproducible, with relative standard deviations (RSDs) of 7.1%, when evaluated with 20 mg L^{-1} creatinine (n = 10). The standard curves in which the intra-run CVs (4.7–6.8%) and inter-run CVs (7.9%) obtained were performed on three different days and exhibited good reproducibility. The method was highly correlated with the conventional spectrophotometric method when real urine samples were evaluated (r^2 = 0.948; n = 15).

1. Introduction

Creatinine is an end-product of creatine metabolism and well recognised as one of the most common analytes providing an assessment of renal function. Because creatinine is eliminated by the kidneys at a constant excretion rate of about 1.6–1.7% per day [1], increasing levels of creatinine in serum or decreasing levels in urine can be used to evaluate kidney function. In addition, its concentration is also employed as a correction factor for fluctuations in urine volume, which is useful for determination of the microalbumin/creatinine ratio [2,3]. Due to its clinical importance, a sensitive and accurate assay for creatinine in blood and urine samples is in demand.

Numerous approaches for creatinine assay have been reported in the literature, such as enzymatic methods [4,5], strip assay [6], HPLC [7], mass spectroscopy [8], capillary zone electrophoresis [9,10], potentiometric biosensors [11–13], flow [14,15] and sequential injection analysis systems [16]. However, the very first and most commonly used method for creatinine analysis is a colourimetric method based on the alkaline picrate of Jaffé reaction [17,18]. Here, creatinine reacts with picric acid under alkaline conditions and subsequently forms an orange-red colour complex, which is monitored by spectrophotometry. Of several methods presently used in clinical laboratories, the methods based on the classical Jaffé reaction continue to be the method of choice in most laboratories [19], despite continuous attempts to overcome the interferences known to exist with this method. For example, without deproteinisation steps, a common practice for overcoming interference in the Jaffé reaction is the two-point kinetic assay, which possesses several advantages in terms of simplicity, rapidity, and ability to run with an automatic analyser [20,21].

In recent research, there has been a growing interest in the concept of micro-total analysis systems (μTAS) or lab-on-a-chip systems. Microfluidic systems offer many advantages over conventional ones, including less reagent consumption, shorter analysis time, and portability. Several applications of microfluidic system have been documented in the area of clinical diagnostics for monitoring and diagnosis of various diseases [22] such as cardiovascular disease [23], diabetes [24], cancer [25,26], and renal disease [27].

Poly(dimethylsiloxane) (PDMS) has been widely adopted as a polymer for microfluidics devices because of its simplicity, ease of fabrication and low cost [28]. The creatinine assay can be adapted to a PDMS flow-through microsystem as described by Grabowska et al. [27]. Absorbance detection is the most universal detection technique, but is classified as an unconventional detection method for microfluidic devices [29]. The general limitations are due to short optical path length and the difficulties in coupling the light into and out of microchannels [29].

This study was initiated to present a simple, low cost and sufficiently sensitive on-chip absorbance detection method for the

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determination of urinary creatinine based on a classical alkaline picrate Jaffé reaction. The optical path length of the microchip could be increased to a 1-cm path length similar to a standard one by means of custom-made flow cell, where the fibre optic cables were horizontally aligned in the opposite direction. The effects of various parameters affecting the assay sensitivity were studied and optimised. We also present validation results for real sample analysis in comparison with the conventional method.

2. Experimental

2.1. Chemicals, reagents and samples

All chemicals were of analytical reagent grade. Creatinine hydrochloride and picric acid were obtained from Sigma. Sodium hydroxide and chemicals for buffer preparation were supplied by Merck (Darmstadt, Germany). Poly(dimethylsiloxane) (PDMS, Sylgard 184) kits were purchased from Dow Corning (USA). Photoresist (SU-8 2100) and developer were supplied by MicroChem (USA). All solutions were prepared in Milli-Q water.

A working Jaffé reagent was prepared daily from a stock solution of 0.05 mol L^{-1} picric acid and 0.5 mol L^{-1} sodium hydroxide at the ratio of 1:1 [20]. Working creatinine standards were freshly prepared by dilution of a stock solution (1 mg mL^{-1} creatinine) in 0.1 N HCl before use.

Unidentified urine samples were collected from the Health Science Operation Service, Faculty of Allied Health Sciences, Chulalongkorn University. The urine specimens were centrifuged at 1500 rpm for 5 min before subjecting the supernatant to analysis with the microfluidic system.

2.2. Apparatus and instrumentation

A miniature fibre optic spectrometer (USB4000) with LS-1-LL tungsten light source was a product of Ocean Optics Inc. (USA). The two-channel syringe pump (Fusion 200) and injection valves (V-451) used in the microfluidic system were products of Chemyx (USA) and Upchurch Scientific (USA), respectively. A spin coater (model WS-400A-6NPP, Laurell technologies Corp.) was used for spin coating of the photoresist onto the silicon substrate for mould fabrication. UV-lithography process was done by MJB4 mask aligner (SUSS microtec, Germany).

The oxygen plasma cleaner (PDC-32G) used for PDMS surface oxidation prior to bonding was a product of Harrick Scientific Corp. (USA). The temperature controller system with 37 ± 0.2 °C accuracy was made locally. The system consisted of a cartridge heater, which provided heat energy, aluminium heating box and thermocouple (temperature sensor). Tygon tubing was obtained from Bio-Rad Laboratories. PTFE tubing with 0.5 mm i.d. and all PEEK connectors used were products of Upchurch Scientific, USA. A UV–vis spectrophotometer (Evolution 600, Thermo Scientific, USA) was used for determination of creatinine based on the conventional Jaffé reaction for comparison to our method.

2.3. PDMS microchip fabrication

A photomask pattern for the PDMS microchip was designed with the L-edit program (version Pro v8.03). The microchannel was 500 μm wide and 100 μm deep. The microchip was divided into three main parts. First, a y-shaped microchannel was used for inlets of reagent and sample; next was the mixing zone containing the microfabricated arrow-head shaped baffles throughout the 8-cm length microchannel. The total calculated volume within the microchannel was 6.5 μL. The multi-physics simulation software, COMSOL, (Fluent Inc., NH, USA) was used to evaluate the mixing efficiency of the microchip with baffles or plain channel.

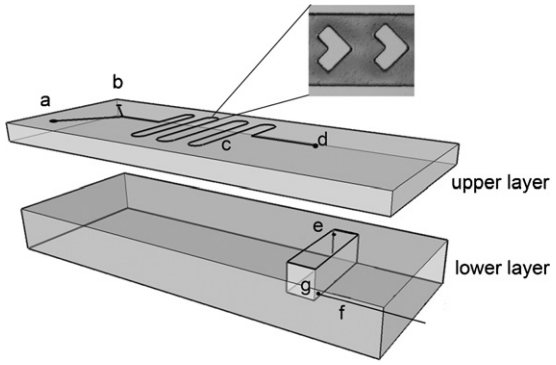

Fig. 1. Schematic layouts of the complete PDMS microchip, and detailed view of the microfabricated arrowed-head shaped baffles at the mixing zone: (a and b) are the inlet holes for alkaline picrate solution and urine sample; (c) is the mixing zone; (d and e) are the outlet and inlet of the upper and lower PDMS layer, respectively; (f) is the outlet for waste solution; (g) is a homemade flow cell made from polystyrene cuvette.

The simulated results demonstrated that using only 15 baffles at the beginning of the mixing zone, the mixing percentage of 88% homogeneity was obtained, whereas those without baffles at the same length could give only 50–60% homogeneity. Therefore, with the current mixer design of total 216 arrowed-head shape baffles throughout the microchip, it was assumed to achieve 100% mixing. Consequently, this type of microchip was employed in all experiments.

The third part was designed to be the area for absorbance detection. The microchip design was then printed onto transparency film and used as a lithography mask. The silicon wafer was coated with 100 μm-thick photoresist (SU-8) by a spin coating technique. To fabricate the PDMS microchip the prepolymer was prepared by mixing a curing agent with PDMS prepolymer at a 1:10 weight ratio, then PDMS was poured onto the SU-8 master mould.

The upper cured PDMS sheet (of 2-mm thickness) with exposed microchannel was peeled off from the master and holes for inlets were drilled in the PDMS chip using metal pipes (1.5 mm i.d). In order to increase the optical path length, a flow cell made from a polystyrene cuvette (1 cm path length, total volume of 90 μL) was incorporated into a lower PDMS microchip (thickness of 6 mm) as shown in Fig. 1. (lower layer). A hole was punched above the detection zone of the lower microchip for receiving the fluid flow from the upper PDMS layer.

To fabricate the complete microchip, an upper and a lower PDMS slab were subjected to surface modification using a plasma cleaner and immediately bonded together. To improve the strength of bonding, the microchip was further heated on a hotplate at 70 °C for 10 min. Tygon tubes (0.8 mm i.d.) were connected to the microchip for inlets and outlets and glued with epoxy resin. Before using, the PDMS microchip was surface-modified by filling with 1 mg mL^{-1} bovine serum albumin overnight to reduce non-specific binding and improve hydrophilicity of the chip.

2.4. Microfluidic system set-up

A schematic diagram of the microfluidic system is shown in Fig. 2. A dual syringe pump (0.098 μm/step, CVs of flow rate accuracy <1%) with two 20-mL plastic syringes (Terumo®, Terumo Corporation) was used to propel the carrier buffer (20 mM phosphate, pH 7.5). Two injection valves, equipped with 100 and 5-μL loops, were used for injection of the alkaline picrate reagent and urine sample, respectively. The PDMS microchip was placed within a temperature controller device made in our laboratory, in which the temperature was maintained at 37 ± 0.2 °C throughout the assay. At the detection zone of the microchip, fibre optic cables

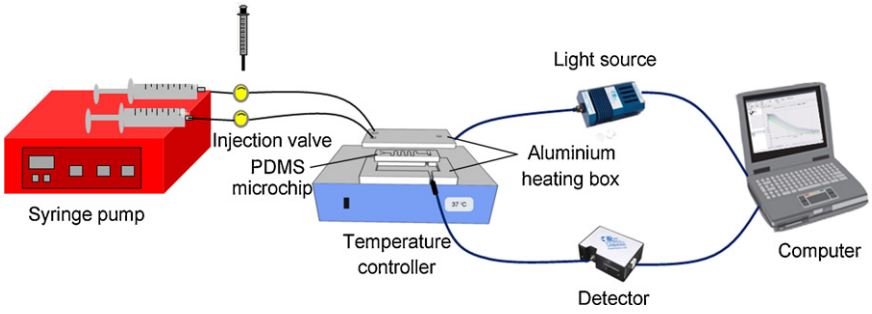

Fig. 2. A schematic diagram of the proposed microfluidic system for creatinine assay.

were horizontally integrated in the opposite direction at 90° to the fluid flow. The first cable was connected to USB4000 spectrometer and another was linked to a light source. Absorbance and spectra changes were monitored using the software provide by the company.

2.5. Assay procedure

Unless otherwise stated, the flow rate of carrier buffer was fixed at 40 μL min^{-1}, where 20 mM phosphate buffer pH 7.5 functioned as both carrier and washing buffer. The temperature controller device was turned on and the buffer flowed through until stable signals were observed. A 100-μL aliquot of alkaline picrate solution and 5 μL of 50-fold diluted urine sample or creatinine standard were injected to the system via the injection valves. After the mixture reached the detection zone, the flow was stopped. Absorbance changes at 510 nm due to accumulation of the reaction complex were recorded for 2 min at 30 s intervals. After each sample, the pump was turned on and the microchip surface and flow cell were washed by means of the carrier buffer until the baseline was gradually reduced to the original signal, which required about 10 min, after which the PDMS microchip device was ready to assay another round of injections.

3. Results and discussion

3.1. Reaction time optimisation

With the Jaffé reaction, a non-specific reaction derived from non-creatinine chromogen can occur late in the reaction and lead to a plateau graph. In addition, the slower reacting substances such as protein, glucose and ascorbic acid can substantially interfere by reducing alkaline picrate to picramate [30]. For this reason, the initial rate of colour development was investigated with real urine samples. Urine was diluted 50-fold before analysis. Results shown in Fig. 3 imply that the optimal time for creatinine determination was 2 min, since a linear response was gained during this period.

3.2. Effect of carrier buffer pH

In the proposed system, the streams of picric acid solution and standard creatinine solution or urine sample were introduced simultaneously by means of separate injection valves. Although creatinine has been recognised to react in alkaline pH, the alkaline picrate solution was not used as the flow carrier because the strong base condition was avoided to continuously affect on the system tubing. In addition, using too alkaline pH condition may lead to carry over effects and require longer time to wash the system. However, we use the carrier buffer in the present work to propel both streams of solution to the microchip. Therefore, the pH of the carrier buffer was studied and optimised since dispersion of reagents and

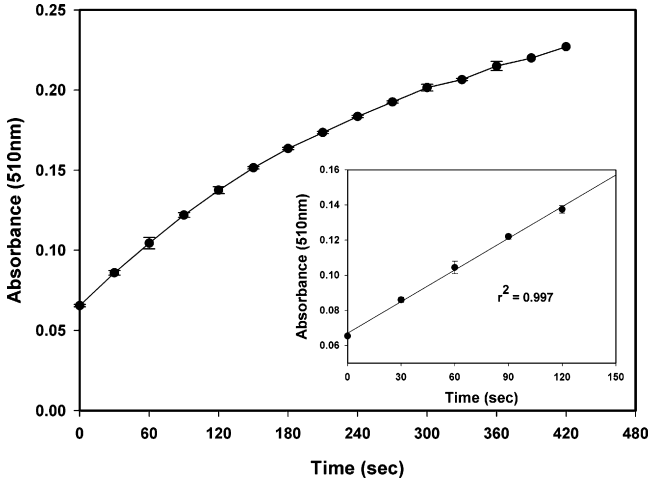

Fig. 3. The absorbance change versus reaction times of urinary creatinine assay by the Jaffé reaction using the proposed system. Each point is the mean value from duplicate assays; the error bar represents the standard deviation.

buffer can influence the colour development. Basically, the buffer functions in maintaining the pH of the Jaffé reaction. The effect of carrier buffer pH on the rate of the Jaffé reaction was studied over the range pH 6–9, with 10 and 40 mg L^{-1} creatinine concentrations.

As shown in Fig. 4, the results demonstrated that the reaction signal increased with pH up to 7.5 when 20 mM phosphate buffer

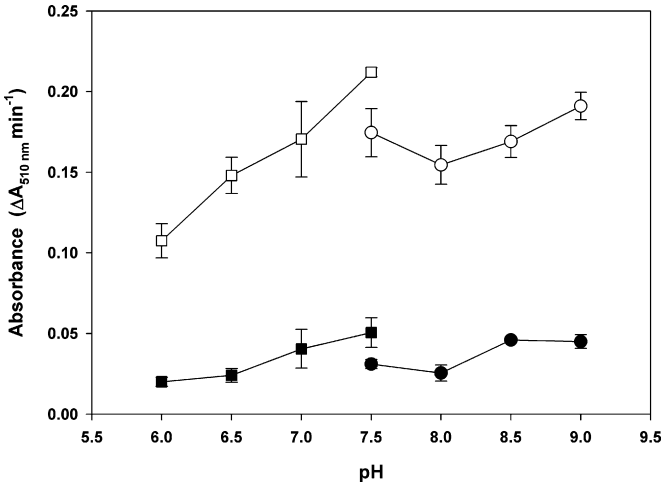

Fig. 4. Effect of buffer and pH to the rate of the Jaffé reaction. Square symbols represent 20 mM phosphate buffer (■) 10 mg L^{-1} creatinine, (□) 40 mg L^{-1} creatinine; circle symbols represent 20 mM Tris–HCl buffer (●) 10 mg L^{-1} creatinine, (○) 40 mg L^{-1} creatinine. Each point is the mean value from duplicate assays; the error bar represents the standard deviation.

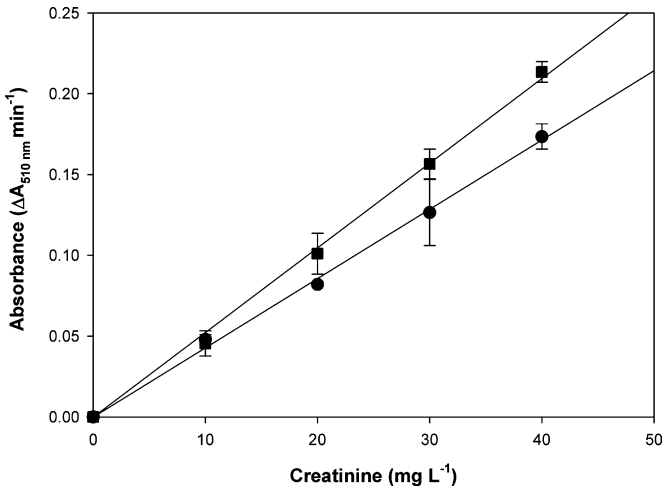

Fig. 5. Effect of temperature on the rate of Jaffé reaction for determination of creatinine. (●) 25 °C; (■) 37 °C. Each point is the mean value from duplicate assays; the error bar represents the standard deviation.

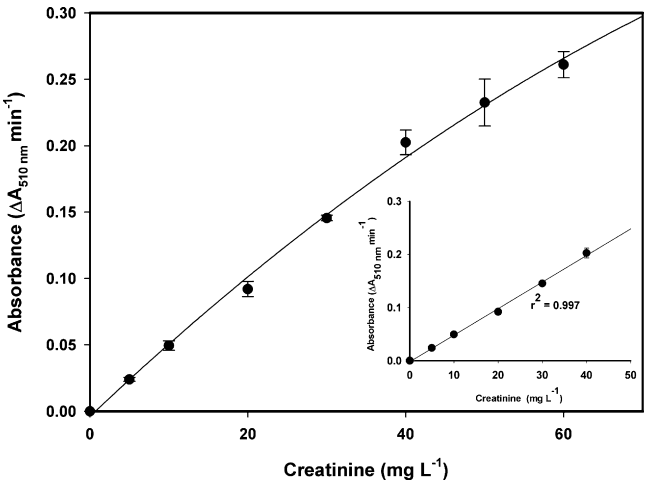

Fig. 6. Standard curve of creatinine by the Jaffé reaction in microfluidic system. Inset, a linear range of creatinine response was achieved at 0–40 mg L^{-1} (r^2 = 0.997). Each point is the mean value from duplicate assays; the error bar represents the standard deviation.

was used. However, it was noticed that the signal was not improved at higher pH while using Tris–HCl buffer. The results indicated that different kinds of buffer have different effects on the reaction rate. For example, similar signals were attained by using phosphate buffer pH 7.5 or Tris–HCl buffer pH 9.0. Therefore, 20 mM phosphate buffer pH 7.5 was chosen for further experiments because its pH is close to neutral.

3.3. Effect of temperature

Extensive studies have established that the rate of colour development in the Jaffé reaction is influenced by temperature [27,31]. In this study, the creatinine calibration curves obtained from different temperatures between 25 and 37 °C were compared. It is apparent that the sensitivity of the reaction is much better at higher temperature, which is in agreement with other reports. The sensitivity gained from performing the reaction at 37 °C was about 1.2-fold higher than that at 25 °C, as shown in Fig. 5. For this reason, we kept the temperature constant at 37 °C during all experiments, by means of the temperature controller.

3.4. Analytical curve

A 100-μL aliquot of alkaline picrate solution and 5 μL of a standard solution of creatinine (0, 5, 10, 20, 30, 40, 50 or 60 mg L^{-1} creatinine) were injected into the system via different injection valves. The standard curve for creatinine is shown in Fig. 6. In this study, the assay range was well fitted in the range of 0–40 mg L^{-1} creatinine (r^2 = 0.997). The detection limit (LOD) was calculated from 10 replicate assays of the blank sample. Based on an S/N = 3, the detection limit obtained for creatinine was 3.3 mg L^{-1}.

Recently, there was a report based on alkaline picrate colour reaction using zone fluidic multichannel kinetic spectrophotometry described by Ohira et al. [32]. The system demonstrated many satisfied performances such as low limit of detection (0.76 mg L^{-1}), and wide linear range (0–250 mg L^{-1} creatinine). However, when comparing to our system in terms of reagent and sample consumptions, the proposed microfluidic system used 2-fold and 40-fold less reagent volume and sample, respectively, than those of the ZF method. According to the cleaner analytical procedures [33,34], minimizing waste generation should be a relevant advantage of our system

3.5. Analysis of spiked urine sample

Urine samples were spiked with creatinine standards to obtain final concentrations of 0, 5, 10 and 20 mg L^{-1}. The creatinine concentration of each urine sample was then determined by the proposed system using the above calibration curve. Each concentration was a duplicate assay. High recovery results were obtained, with 99.30 ± 0.53% to 104.81 ± 0.37% (Table S1 in the supplementary data).

3.6. Precision and reproducibility

The statistical analysis of the reproducibility of the study was calculated from three calibration curves performed on three different days. The method exhibited good reproducibility, as assessed by intra-run CVs (4.7–6.8%) and inter-run CVs (7.9%). In addition, the measuring absorbance signals were found to reproducible for the measurement of 20 mg L^{-1} creatinine with relative standard deviations of 7.1% (n = 10). The results indicate that there are no significant differences between days when using the same chip, which demonstrated not only the reusability of the system but also the practicality of the microchip.

3.7. Interferences

It has been known for many years that the Jaffé reaction has a significant defect in terms of specificity because many compounds, some normally existing in urine samples, have been reported to produce a Jaffé-like chromogen [35]. As reported in the original method, varying degrees of interferences were found from compounds such as glucose, albumin, and carbonyl compounds [30,36].

Table 1
Effects of the tested substances on the creatinine assay using the proposed microfluidic system. A mixture of 30 mg L^{-1} creatinine and interfering substances were determined.

Tested substances	% Recovery
None	100 ± 2.51
6 mg L^{-1} haemoglobin	114.4 ± 9.5
1 mg L^{-1} haemoglobin	101.9 ± 1.3
300 mg L^{-1} human serum albumin	104.8 ± 14
100 mg L^{-1} transferrin	100.0 ± 9.3
1000 mg L^{-1} IgG	112.2 ± 5.3
20 mg L^{-1} IgG	101.8 ± 12

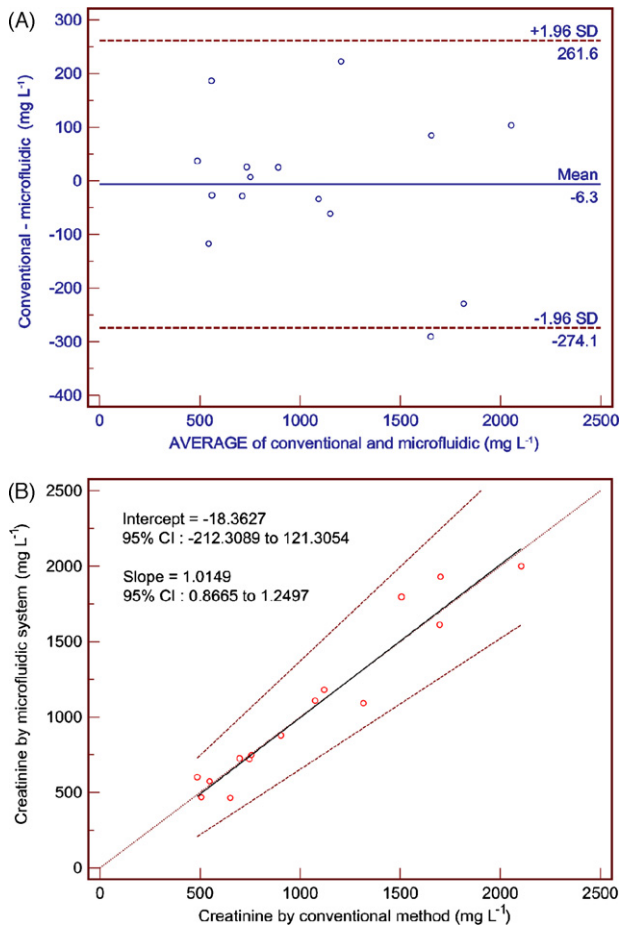

Fig. 7. The microfluidic system and the conventional method were compared for the creatinine assay. Results shown with Bland–Altman bias plot (A) and Passing–Bablok regression analysis (B).

However, interferences from urine samples for creatinine assay were rarely observed [31]. Because it is necessary to dilute urine samples 50–100-fold before analysis interferences can thus be substantially diminished. For example, in case of first morning urine samples, the reference values of creatinine in Thai populations are about 390–2590 and 280–2170 mg L^{-1}, for male and female, respectively. Because the proposed microfluidic system has a working assay range at 0–40 mg L^{-1} of creatinine concentration, average urine samples can be diluted up to 100-fold before subjected to the assay.

Furthermore, a small sample injection (5 μL) and a large volume of detection cell (90 μL) were utilised in our experiment. Since this could lead to a high dilution of sample, the sample was further diluted for about 20-fold. In deed, this has an advantage because the endogenous urine colour possibly contributing to the background absorption or the substances potentially interfering with the assay reaction were significantly diluted out, thus minimizing their interfering effects.

In addition, kinetic assays have been developed in a quest for improved specificity of the Jaffé reaction [20]. However, partial inference from proteins remains in the kinetic method.

With the criteria of a 95–105% recovery range as acceptable, the results demonstrate that 6 mg L^{-1} haemoglobin and 1000 mg L^{-1} IgG significantly interfered in the proposed system (Table 1). In general, these findings confirm the observations reported in the literature. However, lower levels of haemoglobin, IgG or transferrin at 100 mg L^{-1} and albumin at 300 mg L^{-1} were not found to interfere significantly with the assay.

3.8. Determination of creatinine in urine samples

To demonstrate the applicability of the proposed microfluidic system for real sample analysis, urine samples were assayed and results compared with those obtained from the conventional spectrophotometric method. Urine samples ($n = 15$) were diluted 50-fold before assay so the creatinine concentrations fell within the range of the calibration curve. A scatter-plot of the results obtained by both methods is shown in Fig. S1 in the supplementary data ($r^2 = 0.948$, $n = 15$). The regression analysis displayed as $y = 1.023(\pm 0.0008)x - 17.75(\pm 14.43)$.

Both methods were subjected to analyse with Bland–Altman test [37], and the results are shown in Fig. 7A. There is no apparent bias for creatinine determined by the proposed microfluidic system because the differences between the two methods are within mean ± 1.96 SD. The agreement between the microfluidic system and the conventional spectrophotometric methods was also assessed by Passing–Bablok regression [38], as shown in Fig. 7B. The equation for the Passing–Bablok regression line, $y = 1.0149x - 18.3627$ was displayed. According to the 95% confidence interval, the results based on statistical analysis demonstrated that the slope did not significantly differ from 1, whereas the intercept was significantly different from 0. These data suggest that the proposed microfluidic system for determination of urinary creatinine was in agreement with the conventional spectrophotometric method. In addition, a linearity test implied no significant deviation ($p > 0.1$).

4. Conclusions

In this work, a microfluidic system for determination of creatinine has been developed. A universal absorbance detection was performed directly on-chip by utilisation of a portable miniature fibre optic spectrometer. The sufficient sensitivity is required for the proposed system because several folds dilutions of urine sample were achieved before assay, and the small injected sample was unavoidably diluted by the large volume of flow cell. By utilising a custom-made flow cell, these together considerably improved the assay sensitivity due to the standard path length in the microchip being the same as in the conventional spectrometry assay. The microfabricated arrowhead-shaped baffles within the microchannel were used to enhance the mixing efficiency of reagent and sample. In conclusion, several potential advantages appeared to be more pronounced in our system, including simplicity of operation and convenience, low cost of analysis, less reagent and sample consumption, good reproducibility of results, reusability of the microchip, and full portability for in-field analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aca.2009.05.014.

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