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Received January 22, 2013
Revised July 16, 2013
Accepted August 4, 2013

Research Article

Point of care creatinine measurement for diagnosis of renal disease using a disposable microchip

A point-of-care device for the determination of elevated creatinine levels in blood is reported. This device potentially offers a new and simple clinical regime for the determination of creatinine that will give huge time savings and removal of several steps of determination. The test employs a disposable prefilled microchip and the handheld Medimate Multireader®. By optimizing the analytical conditions it was found that the LOD of the proposed method was 87 μM creatinine, close to the normal human serum levels that are in the range of 60 to 100 μM. A statistical analysis of the residual shows a normal distribution, indicating the absence of systematic errors in the proposed method. The test can be used to distinguish patients with renal insufficiency (creatinine levels >100 μM) from healthy persons. Long-term monitoring could furthermore distinguish between acute renal failure and chronic kidney disease by the rate of creatinine concentration rise.

Keywords:
Creatinine / Kidney disease / Microchip / Point-of-care / Screening
DOI 10.1002/elps.201300255

1 Introduction

It is well know that the concentration of some components in blood serum directly reflects human health conditions [1]. Creatinine is an especially interesting analyte, which serves as one of the most widely used markers of renal function. Normal levels of creatinine in human blood are 0.6–1.2 mg/dL (60–110 μM) in serum adult males, and 0.5–1.1 mg/dL (45–90 μM) in serum adult females [2]. These creatinine levels of lower than around 1 mg/dL (100 μM) in healthy adults can increase up to 10 mg/dL (1 mM) in patients with renal failure [3]. A point-of-care (POC) device for the diagnosis of renal disease should therefore be able to determine creatinine concentrations above 100 μM. When it comes to creatinine determination in plasma, isotope dilution (ID)-GC-MS can be considered the primary reference measurement procedure able to provide the "conventional true value." Furthermore HPLC techniques proved to have good performance with respect to ID-GC-MS [4]. Clinical laboratories routinely determine creatinine by Jaffé reaction [5]. However, use of the Jaffé method is an issue of continued debate. Alkaline picrate reacts with creatinine to form a chromogen that absorbs light at 490–520 nm.

Numerous approaches for creatinine assay have been reported in the literature, such as enzymatic methods [6, 7], strip assay [8], HPLC [9], MS [10], CZE [11, 12], potentiometric biosensors [13–15], flow [16, 17], and sequential injection analysis systems [18]. Therefore, the development of an accurate and reliable method for routine creatinine measurements remains an important objective to improve the care of renal patient across the world [19]. There is an increasing interest in the use of CE for the determination of physiologically significant small ionic substances in blood serum, particularly using electrochemical detection because its compatibility with micromachining technologies [20].

Since one of the earliest microfluidic platform reported by Harrison and Manz in 1993 for amino acids [21], the application of micro total analysis systems has rapidly grown over the last decade, due to its promised advantages of small size, less reagents, and the power of integrating other lab procedures, with especial significance in clinical and biomedical field [22]. The development of POC electrophoretic devices based on lab-on-a-chip technology has been a significant contribution in this way [23]. In this context, Wang and Chatrathi reported a microfabricated electrophoresis chip for bioassay of renal markers [24], and García and Henry a microchip CE with pulsed electrochemical detection for the direct detection of renal function [25]. More recently, Yang et al. used a CE microchip with LED induced fluorescence detection for a rapid determination of creatinine in human urine [26]. In this article, we report on the use of the Medimate Multireader® as POC disposable device to detect creatinine levels above 100 μM in human serum. The device is based on electrophoretic separation and conductivity detection and its microfluidic chip has a single opening for sample

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Abbreviation: POC, point-of-care

Colour Online: See the article online to view Figs. 1–5 in colour.

introduction. Operator actions needed are solely the deposition of a sample drop at this opening and insertion of the chip into the Multireader, making the device suitable for the use by untrained operators [23]. In order to develop the analysis conditions for creatinine determination in blood, we studied the effects of sample preparation, electrolyte pH and concentration, and variations in the injection and separation voltages.

2 Materials and methods

2.1 Standards and samples preparation

All reagents used were analytical grade and were purchased from Sigma. Creatinine standard, buffer solutions and all dilutions were prepared with milli-Q water, and stored in a refrigerator at 4°C. The calibration solutions were prepared by appropriately diluting stock solutions.

In all samples 140 mM NaCl was added to obtain the ionic strength of human serum and generate the same stacking effect as in conditions where a blood or serum sample would be applied.

2.2 Apparatus

Figure 1 shows a photograph and schematic of the microfluidic chip. Previously this device has been applied for lithium quantification in blood, and all details on the device and chip as well as the conductivity detection employed can be found in a previous publication [23].

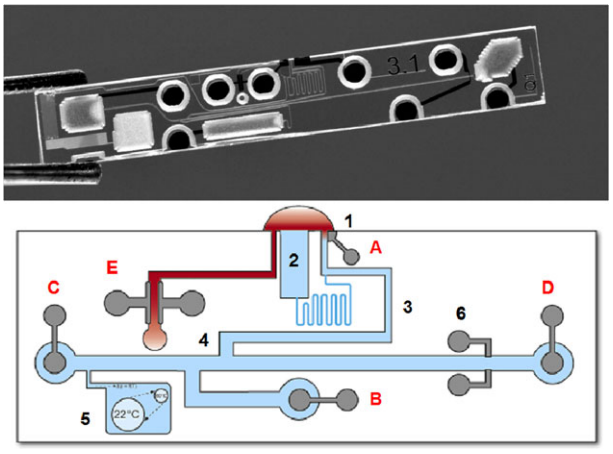

Figure 1. Top: photograph of the microfluidic chip; bottom: Schematic indication of the different functional units (the exact locations slightly differ from the photograph for the sake of clarity). (1) Sample opening with applied sample droplet; (2) evaporation reservoir; (3) injection channel for injection of cations by moving boundary electrophoresis; (4) double-T injector; (5) reservoir with gas bubble for liquid expansion control; (6) conductivity detection electrodes; (A, B) high-voltage injection anode and cathode; (C, D) high-voltage separation anode and cathode; (E) Electrodes for the conductometric determination of the sample conductivity. Reproduced from [23] with permission from The Royal Society of Chemistry.

2.3 Measurement procedure

The measurement procedure is based on the use of a handheld analyzer (Multireader) in which a cartridge with a prefilled totally closed microfluidic chip is inserted. Vacuum filling is employed to fill the closed channel system through the single inlet opening and to leave an air bubble in a specifically designed reservoir to buffer pressure in the chip. Prior to measurement a seal is removed from the sample opening and the sample is applied on top of it (see Fig. 1).

After insertion of the cartridge into the analyzer, the measurement protocol is started during which subsequently injection voltages and separation voltages are applied for a specified time. The analysis process is based on an injection with moving boundary electrophoresis and a separation by CZE. During moving boundary electrophoresis sample (de)stacking can take place due to the difference in conductivity between sample and BGE [23]. In the research presented here, the existing platform was modified to optimize it for creatinine determination. For this purpose the voltage and time protocols for injection and separation as well as the buffer solution were varied.

3 Results and discussion

3.1 Optimization of sample conditions

Sample conditions for the nonblood samples were established after an initial study of the influence of sample ionic strength and pH. Table 1 shows these results in terms of sensitivity (peak area) and CV. In all samples 140 mM NaCl was added to obtain the ionic strength of human serum and generate the same stacking effect as in conditions where a blood or serum sample would be applied. Samples prepared in buffer solution of pH 4.7 and pH 2.0 showed a low CV and good sensitivity compared with the (unbuffered) pH 7 sample solution. Samples buffered at pH 2 showed the best sensitivity, but use of this pH was avoided since it was considered too far removed from physiological conditions and the CV was worse.

3.2 Optimization of measurement conditions

In all electropherograms of test samples containing sodium, to simulate human serum conditions, and creatinine, good resolution was obtained for the creatinine and sodium peaks due to the appreciable difference in electrophoretic mobilities of the two substances. Optimization therefore aimed at obtaining high sensitivity (large peak area) and low CV. The first attempt of creatinine determination was carried out in the same physical (injection and separation voltage and time) and chemical (buffer) conditions as the lithium determination protocol [23]. These preliminary results proved that creatinine determination was not possible, and it was therefore necessary to carry out an optimization of physical and chemical conditions of the measurement process.

Table 1. Results of the initial study investigating the influence of sample matrix preparation on reproducibility and peak area

Creatinine 5 mM				
Sample conditions	Na^+ peak area (A.U.)	RSD (%)	Creatinine peak area (A.U.)	RSD (%)
NaCl 140 mM, pH 7	24 755 ± 4091	16	1073 ± 631	59
Serum	27 029 ± 341	2	2023 ± 178	9
NaCl 140 mM, pH 4.7	26 455 ± 1862	7	4334 ± 633	14
NaCl 140 mM, pH 2	28 346 ± 835	3	6491 ± 530	18

3.2.1 Optimization of chemical conditions

Chemical condition optimization was based on the influence of two parameters of the buffer solution used as background electrolyte: pH and ionic strength (μ). Starting with the original buffer (Buffer 0) that was used for lithium determination, with pH 4.7 and 15 mM ionic strength, a comparative study was carried out with four additional different buffer solutions: buffer A (μ = 15 mM; pH = 4.0); buffer B (μ = 15 mM; pH = 3.5); buffer C (μ = 25 mM; pH = 4.7); and buffer D (μ = 50 mM; pH = 4.7).

First, Fig. 2A, B, and C show the electropherograms using Buffer 0, Buffer A, and Buffer B, respectively, for studying the influence of the pH. It can be observed the most important change between pH 4.7 (Buffer 0) and pH 4.0 (Buffer A), while the electropherograms at pH 4.0 (Buffer A) and 3.5 (Buffer B) are almost identical. The change from Buffer 0 to Buffer A results in two important improvements: improved creatinine peak definition and base line stabilization. We explain the improvement of creatinine peak shape by the shorter separation time and a stabilization of the ionized (+1 ion) fraction of creatinine at pH 4.0. Creatinine has a pKa of 4.7, changing its charge from +1 charge to 0. Small changes in pH around pH 4.7 can therefore strongly affect migration speed and cause additional peak broadening. The increase of the ionized fraction also decreases the migration time, reducing diffusional band broadening. The stabilization of the base line in Buffer A and B with respect to Buffer 0 can be ascribed to the increase of buffer capacity, which increases from 18 mM in Buffer 0 to 30 and 33 mM in Buffer A and B, respectively. The absence of differences in results between Buffer A and Buffer B corroborate this theory.

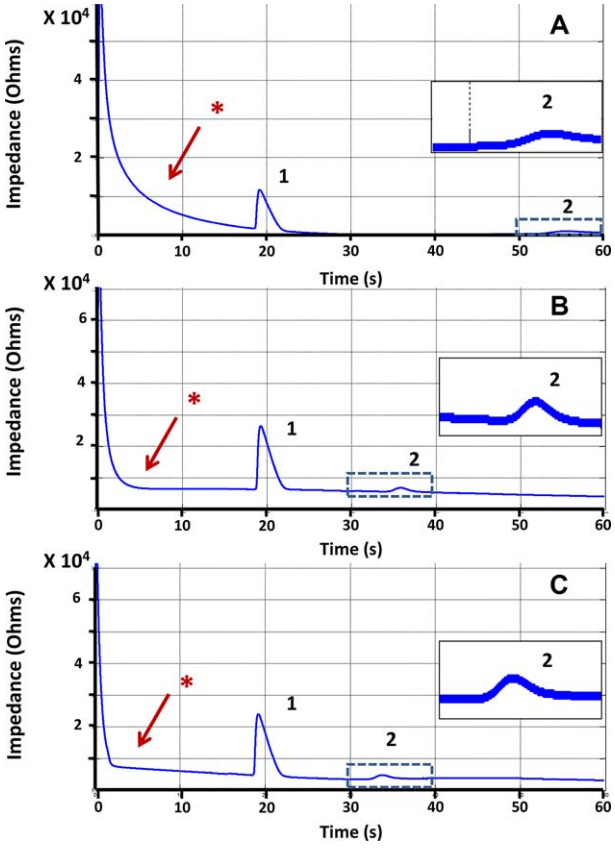

Figure 2. Electropherograms showing base line stabilization (*) and creatinine peak shape improvement due to a change in BGE pH from 4.7 (A), to 4.0 (B), and to 3.6 (C). 1: sodium, 2: creatinine. Sample: creatinine 3 mM, NaCl 140 mM, BGE.

Secondly, the influence of ionic strength was studied, by using Buffer C and Buffer D. It was expected that an increase of ionic strength would increase the amount of creatinine injected due to stacking effect. However, it was found that increasing ionic strength has a big negative influence on the baseline stability, making the reproducibility worse. The increase in noise can be due to the increase in background conductivity, which in both cases results from the higher currents due to the higher ionic strength of Buffer C and D (results not shown). Therefore, Buffer A (pH 4.0, 15 mM) was chosen as the optimal buffer solution for creatinine determination.

3.2.2 Optimization of physical conditions

Instrumental parameters such as separation voltage, injection voltage, injection time, and separation time were optimized. Firstly, the separation parameters were optimized. Figure 3 shows the creatinine peak area as well as the CV at different separation potentials. No appreciable differences in terms of peak area were observed, which is expected since the injection parameters (injection voltage and time) were held constant. Clearly however, significant differences in terms of RSD of the creatinine peak are observed. The improvement of the RSD is ascribed to the increase of the peak height with increasing voltage, caused by the shorter separation times and lower diffusional band broadening. As a result peaks can be

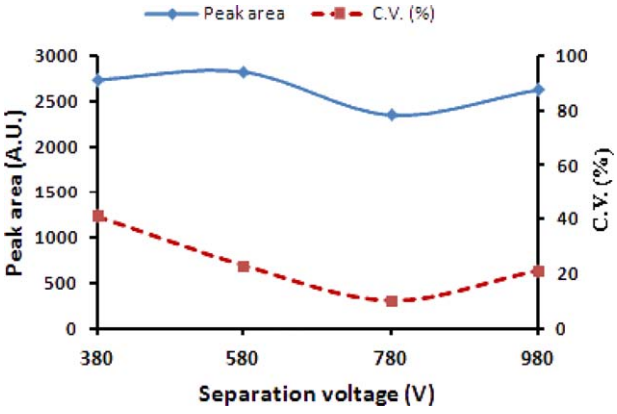

Figure 3. Separation voltage optimization. The peak area and the CV of the creatinine peak are shown as a function of separation voltage. Sample: creatinine 3 mM, NaCl 140 mM, BGE (pH 4.7).

better distinguished from the baseline. A minimal RSD was found at 390 V/cm, which was therefore chosen as the optimal separation voltage. At this separation potential, complete resolution of the sodium and creatinine is obtained in less than 60 s, and this separation time was therefore chosen for all the following optimization steps.

In the next step, the injection parameters, potential conditions, and time, were optimized simultaneously in view of the expected strong dependence between these two variables.

Focusing firstly on the injection potential, Fig. 4A shows how the surface area of the creatinine peak increased with injection voltage at all different injection times. This behavior is as expected, because an increasing field increases the amount of creatinine injected by moving boundary electrophoresis in the injection step. Moreover, Fig. 3B shows that an increasing injection voltage has also a beneficial effect on the RSD. This effect reaches an optimum value around the injection voltage of 680 V, where the lowest RSD is reached for most of injection time studied.

On the other hand, Fig. 3C shows such the highest injection voltage (390 V/cm) achieve the highest sensitivity (peak area) values, however, focusing on the RSD values, Fig. 3D shows that large and illogical RSD variations occur at 390 V/cm between different injection times. These RSD values, produced by the increase of noise can be due to the increase in background conductivity and, hence, the increase of the noise in the system. We explain this effect by bubble formation in the electrode reservoirs when such high potentials are applied, since they will give rise to proportionally larger currents and hence gas production, and therefore discarded the injection voltage of 390 V/cm. Finally, an optimal injection voltage of 340 V/cm was thus selected, while an injection time of 120 s appeared to provide good sensitivity and reproducibility.

Working under the optimized conditions good separations were obtained in terms of resolution and efficiency.

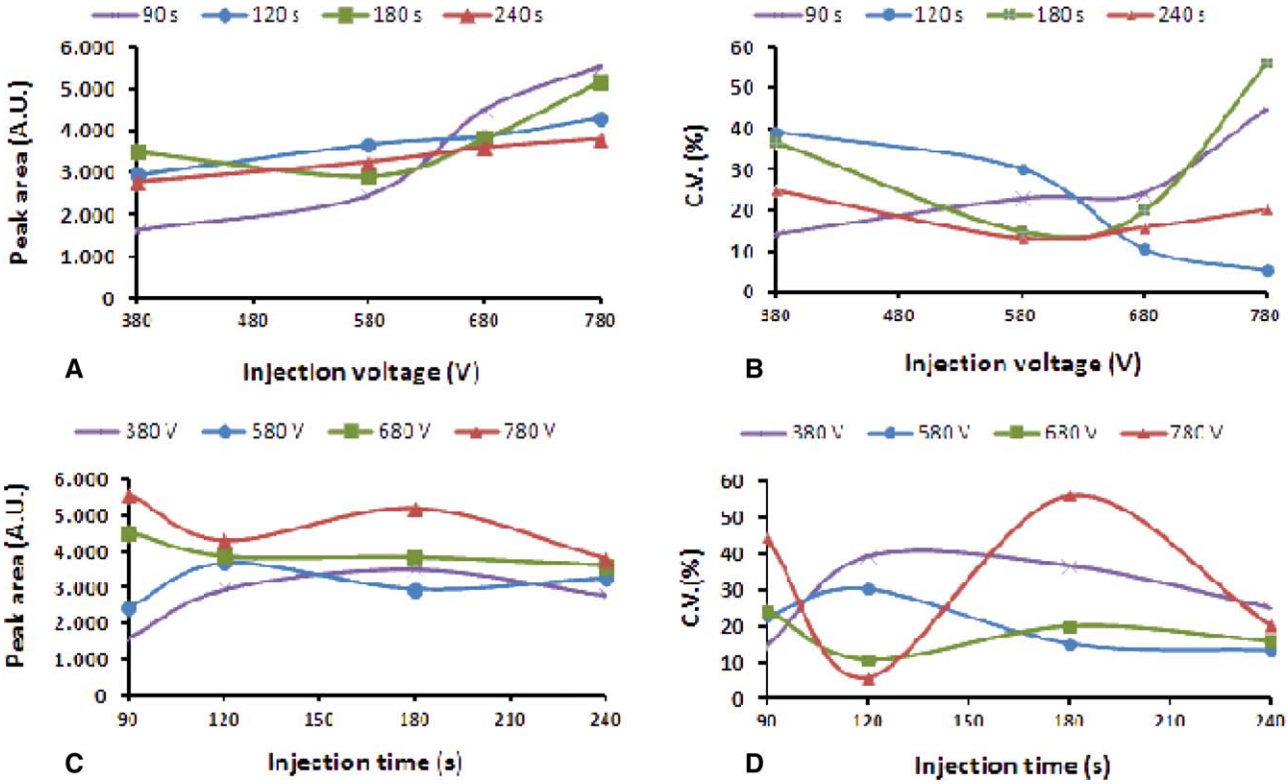

Figure 4. Optimization of injection voltage and time. The peak area and the CV of the creatinine peak are shown; (A and B) effect of injection voltage, respectively, on peak area and CV; (C and D) effect of injection time, respectively, on peak area and CV. Sample: creatinine 3 mM, NaCl 140 mM, BGE (pH 4.7).

Thus, the resolution (R_s) for Li-creatinine was 4.4, and for creatinine-peak 4 (not identified) was 2.8. On the other hand, separation efficiency for creatinine was evaluated by the number of theoretical plates, *N*. A value of ca. 3900 was obtained, which corresponds to a plate height of H = 5 μm approximately.

3.3 Validation of the method and statistical analysis

The analytical figures of merit used to characterize this method included dynamic range, intercept, slope and regression coefficient. Moreover, the LOD and LOQ were included, defined as the concentration of analyte that gives a signal equivalent to the blank signal plus three and ten times the SD of the intercept, S_a, respectively. Thus, under the optimized conditions, the relationship found between the signal (*S*) and the concentration of creatinine (in mM) was $S = (45 \pm 13) + (446 \pm 7)$ [creatinine], for a linear range between 0.06 and 0.46 mM ($R^2 = 0.9911$). Values of LOD = 0.087 mM and LOQ = 0.289 mM were obtained. These results were close to the normal human serum values of 100 μM. As mentioned above, improvements in the features of the method are mainly attributed to lower pH of the buffer solution, producing a higher ionized fraction of the creatinine, as well as the higher buffer capacity of the BGE, which causes baseline stabilization.

Finally, a more extensive and global study (120 measurements) was carried out with the optimized protocol with four different sample concentration levels (0.5, 1.0, 2.0, and 3.0 mM), using five different Medimate Multireaders®, and measuring at three different temperatures (16, 22, and 28°C). The statistical analysis of these results showed that, under different circumstances (concentration ranges, measuring devices, and temperature), systematic errors were not found and the measurement gave consistent data. This study was carried out using a three-way ANOVA test, demonstrating the comparability of results and the reliability of the method with independence of the microchip platform used.

In order to validate the method as a screening method for the classification of samples (distinguishing patients with renal insufficiency from healthy persons), based on qualitative information, three different levels of creatinine have been established. Thus:

(i) Cut-off level at the LOD: 87 μM (corresponding peak area signal: 84 ± 4).
(ii) Threshold: 100 μM (corresponding peak area signal: 90 ± 5).
(iii) Limit of decision: 175 μM (corresponding peak area signal: 123 ± 5).

The limit of decision is statistically defined as the concentration corresponding to the signal of blank plus six times its SD. In this way, serum samples can be classified as clearly negative (signal lower than 80) or clearly positive (signal higher than 128). Signals between 80 and 128 corresponded to inconclusive samples with an associated probability of positive samples. It is important to remark this screening method does not provide quantitative information, but only a binary positive/negative response based on electrophoretic peak area signal. Standards at the three levels of concentration indicated before are used for signal calibrating purposes. Signal zone at the 80–128 range is characterized by the possible presence of false positive and false negative responses.

3.4 Application of the method to the analysis of creatinine in human serum

Preliminary measurements were carried out to demonstrate the applicability of the method to real samples. Human serum samples spiked at three different creatinine concentration levels were analysed from a quantitative point of view. Table 2 shows the results. For serum 1 and serum 2 samples orientate values are given, as the concentrations were below to the LOQ. Serum 3 sample concentration (included its precision) is at the LOQ level, and it can be quantified, as well as serum 4 sample. Serum samples were obtained from patients who had lithium treatment. For this reason we can see a lithium peak together with the sodium and creatinine peak in the electropherograms shown in Fig. 5.

The practical potential of the methodology could be of more interest when applied as a screening method using the signal for classifying samples as positive or negative, according to the principles established in Section 3.3. Thus, used in this way, samples containing different concentrations of creatinine can be classified from the experimental peak signal of the electropherogram, as it is shown in Table 3. Samples containing 60 or 200 μM of creatinine were classified as negative and positive, respectively, at 100% of reliability, where reliability (%) = (100 – percentage of false responses).

Table 2. Results of creatinine measurements in human serum as a quantitative method

Sample	Analyte	Conc. added (μM)	Conc. found (μM)
Serum 1	Creatinine	——	(65)
Serum 2	Creatinine	100	(221)
Serum 3	Creatinine	200	282 ± 7.1
Serum 4	Creatinine	250	316 ± 6.8

Table 3. Results of creatinine measurements in human serum as a screening method

Creatinine concentration (μM)	Peak signal	Result of screening	Reliability (%)
60	71	Negative	100
90	85	Inconclusive	Negative (78%)
100	91	Inconclusive	Negative (57%)
175	122	Inconclusive	Positive (90%)
200	137	Positive	100

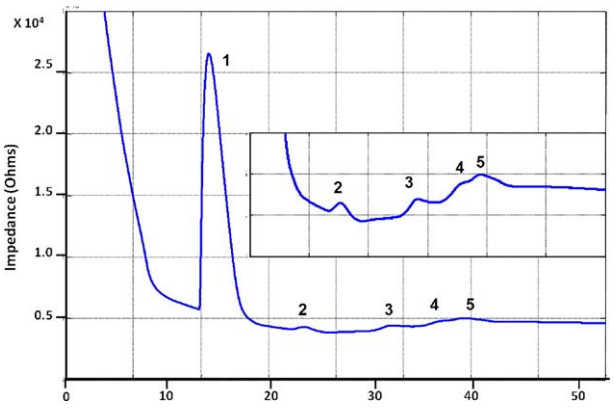

Figure 5. Electropherogram of human serum spiked with creatinine 200 μM. Peaks 1: sodium, 2: lithium, 3: creatinine, 4,5: unknown substances.

Samples containing concentrations of creatinine within the unreliability region (80–228 μM), are classified as inconclusive samples, but with the possibility to include the information of the percentage of reliability and, hence, the probability to be classified as positive or negative samples.

4 Concluding remarks

We have shown that it is possible to use the Medimate Multireader for creatinine determination in human serum. The limits of detection and quantification achieved are at the higher threshold of the normal human levels in blood, ≈ 100 μM. The method can thus be used as screening method for possible kidney problems that result in creatinine levels above 100 μM. This device potentially offers a new and simpler clinical regime for the determination of creatinine that will give huge time savings and removal of several steps previous to determination such as sample preparation. That means to reduce the diagnosis time from hours to minutes in an illness, which can be a question of life-or-death, such as the end-renal stage disease. In the future the measurement will be further validated in a clinical setting.

GO as funded by EFRO is gratefully acknowledged for their funding of the Nirion project in which this project was carried out. The work at Medimate BV was performed in the context of the Nirion project. Finantial support received from the grant CTQ2010-15027 (Spanish Ministry of Economy and Competitiveness) is also acknowledged.

The authors have declared no conflict of interest.

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