Creatinine in urine - a method comparison

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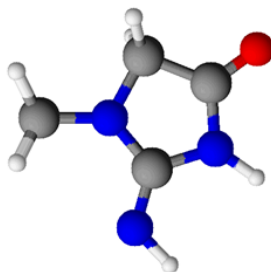[1]Institute of Forensic Medicine, University of Bern, Switzerland

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Three different methods for the determination of creatinine concentrations in urine are carefully investigated. LC-MS/MS proved to be advantageous compared to a spectrophotometric or point-of-care testing based analysis method.

Abstract

Drug screening in urine is widely applied in forensic toxicology. Contrary to blood analysis, excessive or reduced fluid intake can substantially alter the concentration of substances in urine. As a standard to detect urinary dilution, creatinine concentrations are analyzed. A sample with a concentration below 20 mg/dL is generally defined as too diluted to provide a valid result in abstinence control samples. This work investigates the potential of three different methods for the determination of creatinine concentrations in urine samples: A ZIC-HILIC based LC-MS/MS method, a spectrophotometric method on an AU 480 chemistry system, and a portable, chemical reaction based, point-of-care testing device was compared by measuring 200 urine samples. When comparing the two laboratory methods, LC-MS/MS and spectrophotometry, a mean difference of 3.7 ± 14 mg/dL was found, indicating that the spectrophotometric method is slightly overestimating the creatinine concentration. When comparing the LC-MS/MS method with the point-of-care testing device, a mean concentration difference within the calibration range for POCT (>20 mg/dL (excluding 16 samples) and <500 mg/dL (excluding 4 samples)) of 21 ± 37 mg/dL was found, indicating that the point-of-care testing device overestimates the measured creatinine concentration. A point-of-care testing device as used during this study can provide valuable information for on-site analysis. However, reported concentrations above 500 mg/dL should be further evaluated e.g. by dilution of the sample.

Introduction

As excessive or reduced fluid intake can substantially alter the concentration of substances in urine, the necessity of investigating the urine's dilution when measuring drugs of abuse (DOA) or performing ethyl glucuronide testing is widely known (1, 2). This is usually done by measuring creatinine, the internal anhydride of creatine (3). Based on a normal range of creatinine excretion from 14 to 26 mg/kg per day for men and 11 to 20 mg/kg per day for women, the urine's creatinine concentration can vary massively, because the individual urinary creatinine output correlates better with muscle mass than with body weight (2). An obese person is thereby expected to have a lower urinary creatinine excretion than an athletic individual with the same or even lower body mass (3). Nevertheless, the measured urinary creatinine concentration is frequently used to provide information about the dilution, independent of the individual body composition of the donor: According to the European guidelines for workplace drug testing in urine, samples with a creatinine concentration below 22.6 mg/dL should be considered as dilute (4). Dilution of the urine may lead to an underestimation or non-detectability of the drug concentration (2).

In this study, we provide a comparison between two laboratory methods for the urine's creatinine analysis: liquid chromatography - mass spectrometry and spectrophotometry. In parallel, a point-of-care testing (POCT) device based on the oxidation of an indicator dye by a creatinine/copper complex and a hydroperoxide is evaluated by measuring the same urine samples as with the two laboratory methods. Knowledge about the reliability of such a portable device may help the investigator on-site, e.g. during roadside testing, to decide if an additional blood test, to detect drugs of abuse, is advisable. We refrained from testing the urine samples with an enzymatic assay using the Trinder Reaction, as various substances such as N-acetylcysteine (NAC), calcium dobesilate, acetaminophen and the metabolite N-acetyl-p-benzoquinone, metamizole and the metabolites 4-aminoantipyridine (4-AAP) and 4-methylaminoantipyrine (4-MAP) cause falsified results for Trinder based assays (5-7). Acetaminophen and metamizole are often present in the studied population.

Materials and methods

Reagents

Creatinine and creatinine-d_3 were obtained from Sigma-Aldrich (St Louis, USA). Ammonium acetate fractopur® was ordered from Merck (Darmstadt, Germany). Acetonitrile (p.a.) was bought from Acros Organics (Geel, Belgium). Water was produced with a Milli-Q water system from Millipore (Billerica, USA). Creatinine reagent (Creatinine urine kit OSR 6178) for spectrophotometric calibration was obtained from Beckman Coulter (Nyon, Switzerland). A liquid assayed urine control for spectrometry (MAS Urichem Trak) was obtained from Thermo Fischer Scientific (Reinach, Switzerland). The P.I.A.[2] POCT device and creatinine select plate tests were provided by Protzek (Lörrach, Germany).

Study design

89 urine samples used in this study were obtained from eight healthy volunteers participating in a drinking study (8). Further 111 samples reached the institute of forensic medicine for routine analysis (driving under the influence of drugs) and were analyzed for their creatinine concentration. The determination of the creatinine concentration by spectrophotometry represents a standard procedure which is generally performed for all urine samples sent to our laboratory. All samples were anonymized prior to analysis by using a specific sample identification code. In total 200 urine samples were analyzed for their creatinine concentration with three different methods: LC-MS/MS, spectrophotometry, and a chemical reaction based POCT device.

Determination of creatinine by LC-MS/MS

Sample preparation, analysis, and validation has recently been described in detail (8). Briefly, 50 µL of urine and 150 µL acetonitrile containing the internal standard (creatinine-d_3) were shaken for several minutes. After centrifugation, the supernatant was taken and evaporated to dryness. Prior to instrumental analysis, the sample was reconstituted in 400 µL of reconstitution buffer. Chromatographic separation was performed with a SeQuant® ZIC®-HILIC 3.5 µm, 100 Å, 150 x 2.1 mm PEEK coated HPLC column (Merck, Darmstadt, Germany), heated at 30 °C, with a flow rate of 0.3 mL/min. Mobile phase A consisted of a 5 mM ammonium acetate buffer at pH 5.72 with 0.1% formic acid, mobile phase B consisted of acetonitrile with 0.1% formic acid. The following 14 min gradient was used: 0 to 1 min, 95% B;1 to 4.5 min, 95% to 20% B linear; 4.5 to 9 min, 20% B; 9 to 10 min 20% to 95% B linear; 10 to 14 min, 95% B. Mass spectrometric detection was performed on a Sciex 5500 QTrap system, operated in electrospray negative SRM mode (monitored transitions for crea: quantifier: 112→68, qualifier: 112→41, internal standard signal crea-d_3: 115→42). Creatinine eluted from the column with a retention time of 5 minutes. Calibration was performed with a five point calibration prepared in solvent (2, 10, 30, 60, and 250 mg/dL), using a linear calibration model with weighting $1/x^2$. The correlation coefficient (R^2) from least square regression were at least 0.9990. Four quality control samples (2, 10, 20, and 130 mg/dL) were used in each run. Samples with concentrations above the highest calibrator were reinjected after dilution. Mean intra-assay accuracy by measuring six samples of each quality control was 98.8-101.7%, and mean imprecision was 0.7-2.4%. Mean inter-assay accuracy from three series (n=3) with six samples of each quality control in each series was 98.9-101.4%, and mean imprecision was 0.6-2.5%. Furthermore, extraction efficiency was determined to be 55 ± 13 %, the recovery was 53 ± 11 %, and the matrix effects were 96 ± 12 %. The extracted quality control samples proved to be stable after storage for 72 h in the autosampler, after three freeze thaw cycles, and after storage for 7 days at 4 °C.

Determination of creatinine by spectrophotometry

Spectrophotometric determination of creatinine was performed on a Beckman Coulter AU 480 (Nyon, Switzerland). The colorimetric method is based on a kinetic test (Jaffe-reaction), whereby creatinine forms a complex with picric acid, as the deviation in absorption at 520/800 nm is

proportional to the creatinine concentration within the sample (9, 10). The determination is based on a one-point calibration at 153 mg/dL, which is forced through zero. Commercially available quality control urine with a creatinine concentration of 57 mg/dL (acceptable deviation <20%) was included. According to manufacturer specification, the creatinine test shows linearity between 1 to 400 mg/dL (10). A seven point linearity test (0, 10, 20, 50, 100, 200, 300, 400 mg/dL) in our laboratory revealed a correlation coefficient (R^2) from least square regression of 0.9998. Based on repetitive measurements of the quality control sample during two months (n=17), mean imprecision was found to be 6.1%.

Determination of creatinine by POCT

POCT was performed on lateral flow, creatinine select plate tests with an automatic P.I.A[2] readout system from Protzek (Lörrach, Germany), see figure 1. The test is a chemical reaction based device, using copper ions for complexation of the creatinine and a hydroperoxide for the oxidation of an indicator dye for color development of the test field. The calibration model for the determination of the creatinine concentration is based on a saturation curve by comparing the peak-volume (brightness of the reaction pad mounted on a lateral flow device) with the spiked concentration, which is established by measuring eight samples with different concentrations (0, 20, 40, 75, 125, 200, 300, and 500 mg/dL) (figure 2). Accuracy measurements by comparing 24 human urine samples with spectrophotometric measurements revealed a mean accuracy of 112.5% and a correlation coefficient (R^2) of 0.9453. Precision measurements during five days with a low (50 mg/dL) and a high (100 mg/dL) creatinine concentration resulted in a mean imprecision of 10% and 5.7%, respectively. To investigate potential interferences, various substances such as glucose (100 mg/dL), uric acid (100 mg/dL), urea (3000 mg/dL), sodium chloride (1000 mg/dL), and proteins (BSA) (1000 mg/dL) were spiked to a 100 mg/dL creatinine stock solution in triplicate, whereby no influence on the test result was observed (mean imprecision <8%). For sample acquisition the following steps were performed: The provided buffer solution (400 μl, provided with the test cartridge) was mixed with 100 μL of urine, by using a Gilson Pipetman® 200M (systematic error ±1.00 μL, random error ≤0.26 μL). The mixture was shaken for ten seconds at 1,500 rpm using a microtube shaker. Afterwards, 100 μL of the mixture were pipetted on the plate test and measured by automatic photometric readout on the P.I.A.[2] system after three minutes. The result was provided in mg/dL of creatinine with a measurement window of 20 to 500 mg/dL. Alternatively, the device can be used in a direct interpretation mode, as shown in table 1 (11). Each sample was analyzed by a single measurement.

Results and discussion

LC-MS/MS vs. spectrophotometry

When comparing the two laboratory methods, a Bland-Altman plot revealed a mean difference of -3.7 ± 14.3 mg/dL, indicating that the spectrophotometric method is overestimating the creatinine concentration, see figure 3. A one sample t-test reveals a P value of 1.61E-4, showing that the

measured difference between the two methods is highly significant, which indicates the presence of a fixed bias. Linear regression analysis reveals a slope with y = 0.9732x + 6.85 and a R^2 value of 0.9683, see figure 4. This results in an average systematic error of 3.7 mg/dL (y=0.9732$\overline{x}$ + 6.85, whereby $\overline{x}$ for the LC-MS/MS measurement equals 116.90 mg/dL) for the spectrophotometric measurement, which is in agreement with the previously mentioned mean difference of 3.7 ± 14.3 mg/dL from the Bland-Altman plot. When comparing the LC-MS/MS results close to the cutoff concentration (30 mg/dL) for diluted urine, from 0 to 100 mg /dL (with respect to the LC-MS/MS result), linear regression analysis results in a slope of y=1.1484x – 2.80 and a R^2 value of 0.9435, see figure 5. This indicates that the correlation coefficient between the two methods decreases as the observed relative concentration differences increase, when only low creatinine concentrations are investigated. A Bland-Altman plot revealed a mean difference of -5.0 ± 9.0 mg/dL for samples from 0 to 100 mg/dL. Reanalysis of ten randomly selected urine samples resulted in a mean difference of -10 ± 17 mg/dL for the LC-MS/MS analysis compared to -29 ± 13 mg/dL for the spectrophotometric analysis. Spiking of these ten urine samples with an additional 100 mg/dL of creatinine in order to investigate matrix effects resulted with an additional creatinine concentration of 105 ± 9 mg/dL for the LC-MS/MS measurement and 104 ± 11 mg/dL for the spectrophotometric measurement.

When analyzing 20 urine samples in order to compare LC-MS/MS and the spectrophotometric method, the group of Derezinski et al. found a high correlation with a R^2 value of 0.9844 between both methods. In 80% of the tested samples, the Jaffe method demonstrated an overestimation, by 4.6% on average (12). Askenazi et al. found significantly lower urinary creatinine concentrations if samples were measured by the spectrophotometric Jaffe method compared to LC-MS/MS. The observed differences were however less than any clinically meaningful difference (13). A potential reason for this difference could be the preparation of calibration samples. It has to be mentioned, that a LC-MS/MS based method has less interfering substances and shows a higher specificity when it comes to creatinine analysis. This is mainly owed to the chromatographic separation of substances. On the other hand, numerous interferences such as acetone, cefazolin, glucose, bilirubin and lipids were found to influence the outcome of a spectrophotometric measurement (12).

LC-MS/MS vs. POCT

When comparing the LC-MS/MS method with the POCT device, a Bland-Altman plot within the calibration range for POCT (>20 mg/dL (excluding 16 samples) and <500 mg/dL (excluding 4)) revealed a mean difference of -20.7 ± 36.7 mg/dL creatinine, indicating that the POCT device is overestimating the creatinine concentration, see figure 6. With outlier elimination (n=20, <20 and >500), one sample t-test reveals a P value of 1.25E-12, showing that the measured difference between the two methods is significant, thus having a fixed bias. Linear regression analysis reveals a slope with y = 1.1513x + 1.80 and an R^2 value of 0.8631, see figure 7. This reveals an average systematic error of 20.7 mg/dL (y=1.1513$\overline{x}$ + 1.80, whereby $\overline{x}$ for the LC-MS/MS measurement

equals 124.61 mg/dL) for the POCT device, which is in agreement with the previously mentioned mean difference of 20.7 ± 36.7 mg/dL from the Bland-Altman plot. Based on the observed data, it is recommended to repeat measurements at the upper range of the scale (>500 mg/dL, n=4), as they proved to be invalid test results. Samples with a creatinine concentration <20 mg/dL (measured by POCT), accounting for dilute urine, were reliable in 15 out of 16 cases. In one case the POCT device found a creatinine concentration below 20 mg/dL, whereas the LC-MS/MS determination revealed a concentration of 41 mg/dL. Based on literature research, a direct comparison with another portable device for the determination of creatinine in urine is not possible. The currently available POCT devices for creatinine analysis such as the i-STAT (Abbott Point-of-Care, UK) and the Reflotron Plus (Roche Diagnostics, Switzerland) are intended to measure creatinine in whole blood, serum or plasma (14). Based on a review article by Mark DS Shephard, currently available POCT devices for creatinine measurements in blood can be divided into two types: blood gas analyzers such as the i-STAT and "non blood gas" analyzers such as the Reflotron Plus (15).

Conclusion

The observed differences between the three methods can be seen as significant (based on the one sample t-test). As expected, the two laboratory methods LC-MS/MS and spectrophotometry are reliable and provide creatinine concentrations close to each other. LC-MS/MS measurements showed the highest reproducibility. Depending on the liquid chromatography used, creatinine determination can be implemented, within-run, in an already existing LC-MS/MS method for drugs-of-abuse testing. When comparing the POCT device with the LC-MS/MS method, differences in creatinine concentration are more distinct. However, with respect to diluted urine, 15 out of 16 samples with a creatinine concentration below 20 mg/mL (measured by POCT) were identified correctly. It was therefore found that the POCT device may provide a useful tool for the on-site testing of creatinine concentrations. Especially the fact that the device interprets the test outcome automatically was appreciated. However, particular attention has to be paid when creatinine values above 500 mg/dL are reported (4 out of 200 samples, which represents 2%). In this case, a repetition of the measurement is recommended.

Conflict of interest

The authors declare that they have no conflict of interest

Compliance with ethical standards

The participants in the drinking study gave informed consent for the analysis of creatinine in the collected samples. Creatinine analysis of samples reaching the institute for routine analysis is a standard procedure. All samples were anonymized prior to analysis by using a specific sample identification code.

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Tab. 1 Direct interpretation of creatinine results based on the P.I.A.[2] device.

Creatinine [mg/dL]	Category	Interpretation
<20	Invalid	Creatinine concentration is too low. Measurement has to be repeated at a different time point.
20-50	Low	The sample is diluted. Actual drug concentration might be higher
50-150	Normal	Creatinine concentration in normal range
150-500	High	Urine is concentrated. Actual drug concentration might be lower
>500	Very high	Very concentrated sample. Actual drug concentration might be much lower.

Figure caption:

Figure 1 Automatic P.I.A.2 readout system from Protzek with a creatinine test cartridge, a microtube, and a 100 µL pipette for the dilution of the urine.
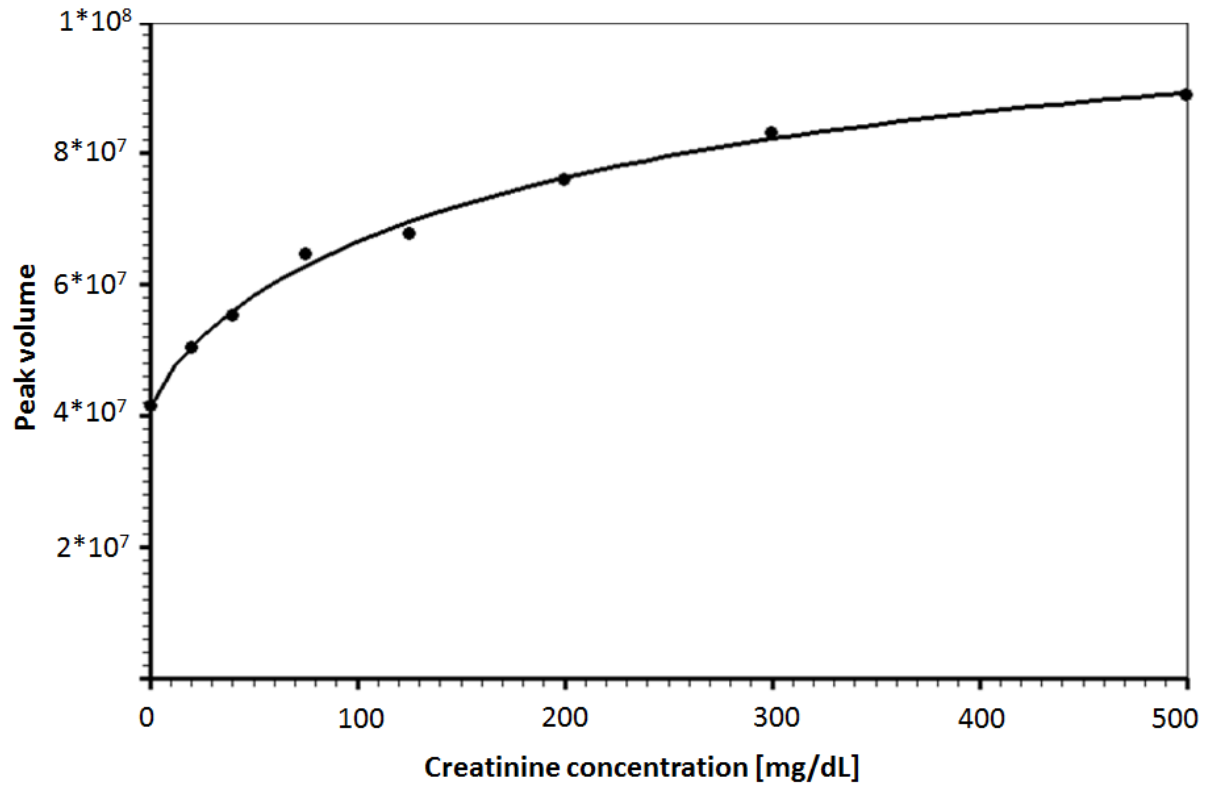
Figure 2 Calibration curve for the POCT device based on a saturation curve established by comparing the peak volume (brightness of the pad) with the spiked creatinine concentration within the sample. (This figure was provided by courtesy of Protzek GmbH)
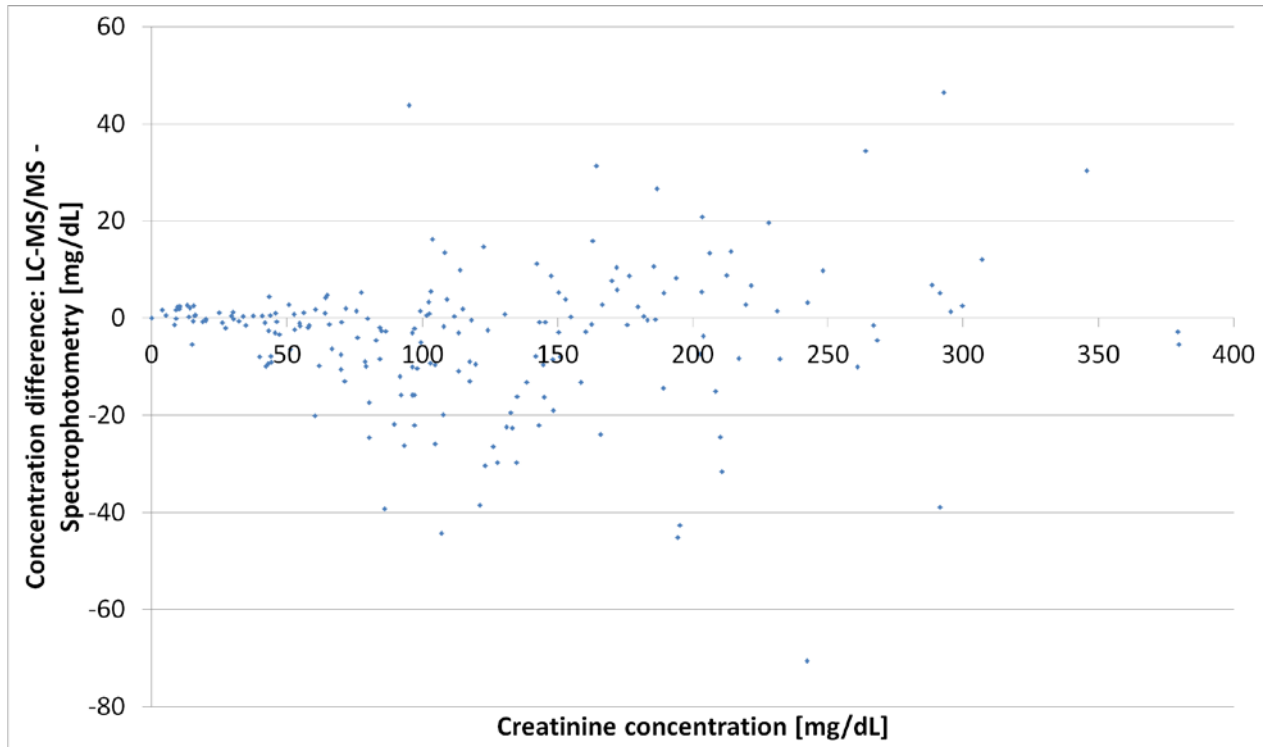
Figure 3 Bland-Altman plot: S(x,y)= ((0.5×(S1+S2), S1-S2)), whereby S1 is represented by the LC-MS/MS concentration and S2 by the spectrophotometric AU 480 analyzer concentration.
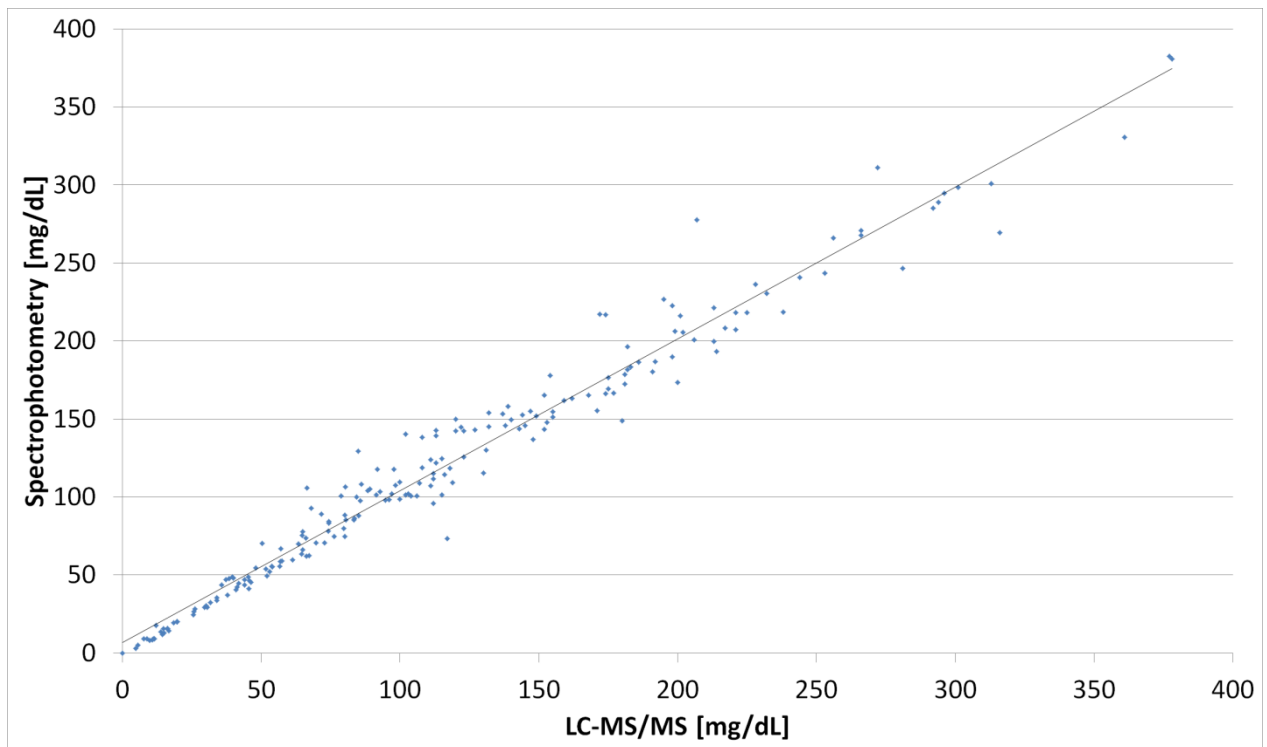

Figure 4 Comparison plot between the spectrophotometric method and the LC-MS/MS method. Linear regression reveals a correlation coefficient R^2 of 0.9683 with y = 0.9732x + 6.85
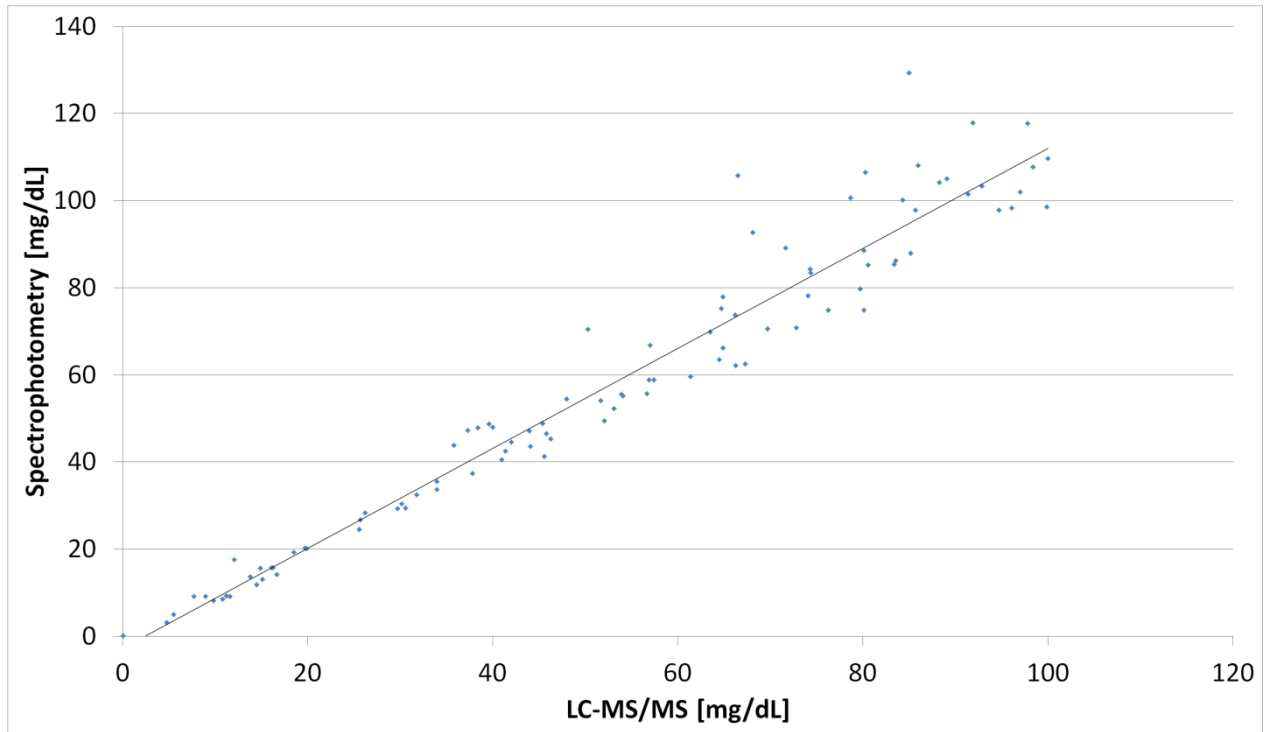
Figure 5 Comparison plot between the spectrophotometric method and the LC-MS/MS method from 0 to 100 mg/dL. Linear regression reveals a correlation coefficient R^2 of 0.9435 with y=1.1484x - 2.80
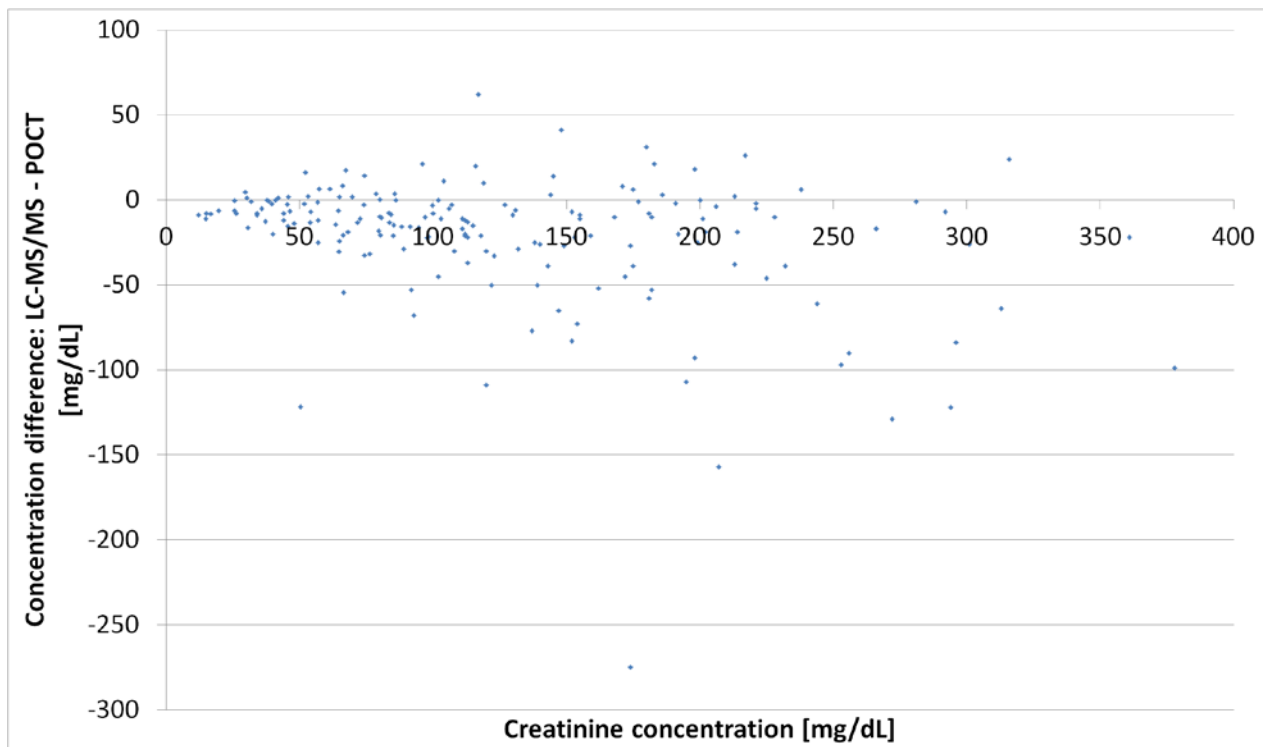

Figure 6 Bland-Altman plot: $S(x,y)= ((S1,S1-S2))$, whereby S1 is represented by the LC-MS/MS

concentration and S2 by the concentration determined by the POCT device. Samples are presented within the calibration range for POCT (>20 mg/dL (excluding 16 samples) and <500 mg/dL (excluding 4 samples))
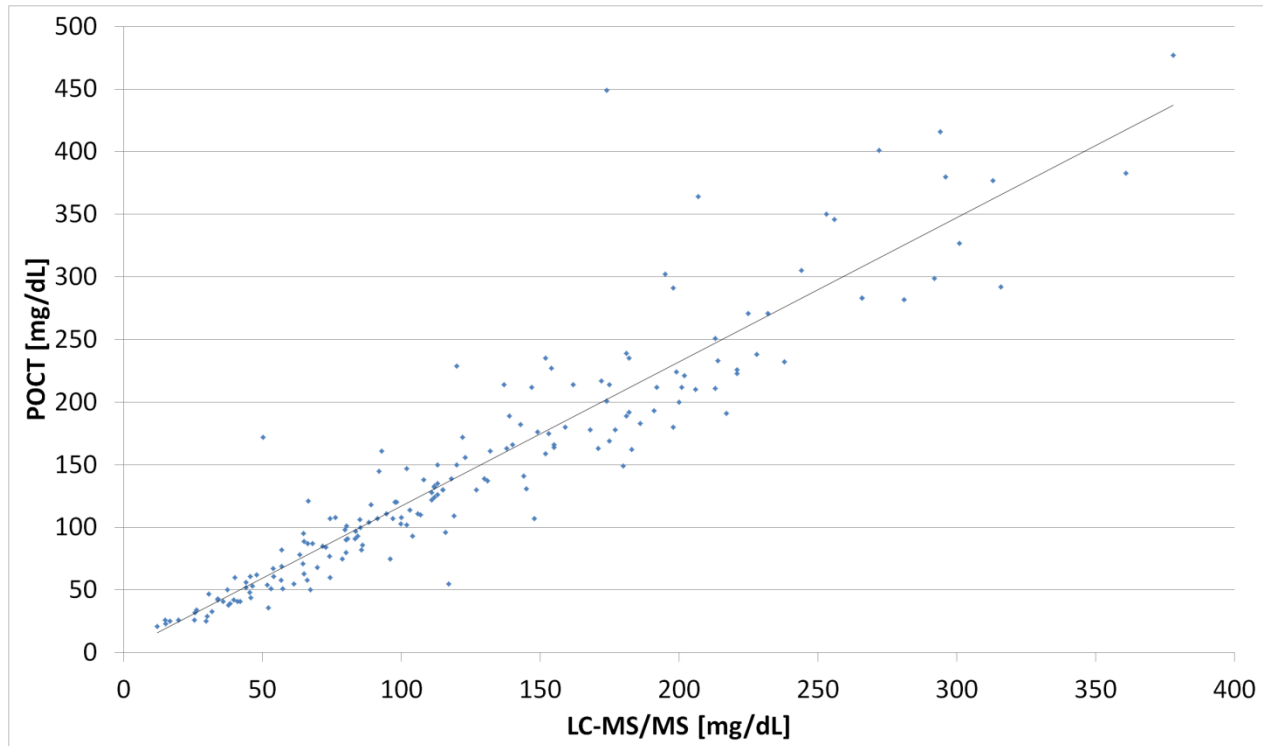

Figure 7 Comparison plot between the POCT device and the LC-MS/MS method. Linear regression reveals a correlation coefficient R^2 of 0.8631 with $y = 1.1513x + 1.80$. Samples are presented within the calibration range for POCT (>20 mg/dL (excluding 16 samples) and <500 mg/dL (excluding 4 samples))