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Plasma creatinine determination in mice and rats: An enzymatic method compares favorably with a high-performance liquid chromatography assay

A Keppler[1,7], N Gretz[1,7], R Schmidt[2], H-M Kloetzer[3], H-J Groene[4], B Lelongt[5], M Meyer[1], M Sadick[6], J Pill[3]

[1]Medical Research Center, University of Heidelberg, Theodor-Kutzer-Ufer, Mannheim, Germany; [2]Department of Clinical Chemistry, Klinikum Ingolstadt, Krumenauer, Ingolstadt, Germany; [3]Roche Diagnostics GmbH, Sandhofer, Mannheim, Germany; [4]Department of Cellular and Molecular Pathology, German Cancer Research Center, Im Neuenheimer Feld 280, Heidelberg, Germany; [5]INSERM U. 489, Hôpital Tenon, 4, rue de la Chine, Paris, France and [6]Department of Radiology, University Hospital Mannheim, University of Heidelberg, Theodor-Kutzer-Ufer, Mannheim, Germany

The use of the colorimetric Jaffé method for the measurement of creatinine in mouse and rat plasma has been criticized as prior studies have shown a dramatic overestimation. We compared a colorimetric picric acid, an enzymatic, and a high-performance liquid chromatography (HPLC) method to assess their appropriateness for routine measurements of creatinine in plasma of healthy and diseased mice ($n = 61$) and rats ($n = 56$). For the colorimetric Jaffé method a pronounced overestimation is confirmed. Additionally the method showed interference with hemoglobin already in a very low, non-visible concentration range in rat plasma. The enzymatic measurement demonstrated a hemoglobin interference in mice, only when hemolysis was visible. The comparison between HPLC and the enzymatic measurement gave a good agreement between both methods in both species. Therefore the enzymatic method fulfills the requirements for a routine screening test for plasma creatinine in healthy as well as diseased mice and rats over a broad concentration range.

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Correspondence: *J Pill, Roche Diagnostics GmbH, Sandhofer Str. 116, D-68305 Mannheim, Germany. E-mail: johannes.pill@roche.com*

[7]These authors contributed equally to this work

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Plasma or serum creatinine is the routinely used marker for the assessment of renal function. It is measured colorimetrically either by direct color formation with picric acid or enzymatically. The reaction of creatinine with picric acid was introduced by Jaffé in 1886.[1] Advantages of this method are its cost effectiveness and ease in performing. The lack of sensitivity and specificity owing to reactions with various endogenous and exogenous chromogenes, such as bilirubin, glucose, or cephalosporins, is a drawback of this method.[2]

Another possibility for measuring plasma creatinine is its enzymatic determination.[3] In this assay, creatinine and derived metabolites are converted by the combined use of creatininase, creatinase, and sarcosine oxidase. Liberated hydrogen peroxide is used to form a colorimetric indicator.

In 1985 Meyer *et al.*[4] reported that, in mouse serum, chromogens cause a fivefold overestimation of serum creatinine when the picric acid assay is compared to high-performance liquid chromatography (HPLC). Notwithstanding this finding, the colorimetric method is still frequently used.[5,6] By use of modified HPLC the dramatic overestimation of creatinine in serum of mice has been confirmed.[7,8] HPLC therefore has been recommended as a precise method to measure creatinine in various species.[9,10] However, the HPLC assay is very time consuming because of the sample preparation and therefore not routinely used to determine serum creatinine.

The objectives of the present study were (i) to compare creatinine concentrations obtained with the Jaffé, enzymatic and HPLC analysis methods in healthy as well as diseased mice and rats; (ii) to evaluate the recovery rate of creatinine spikes; and (iii) to analyze the interference of hemoglobin on the measurement of creatinine with the enzymatic and colorimetric method.

RESULTS

Comparison of HPLC, enzymatic, and colorimetric measurement of creatinine in mouse and rat plasma

Endogenous and spiked creatinine (0.4 mg/dl creatinine added) was measured in plasma samples of mice and rats

(Figure 1) by using HPLC, enzymatic, and colorimetric methods. Creatinine in plasma samples from healthy as well as diseased animals covered a wide concentration range (mice: 0.06–2.72 mg/dl, rats: 0.25–3.09 mg/dl, determined enzymatically). Comparison of the enzymatic and HPLC method by scatter plots revealed a good agreement in both species (Figure 1). In contrast the colorimetric method was consistently overestimating creatinine concentrations in both species, an effect that was increasing with higher creatinine values. The comparison between the colorimetric and enzymatic measurement gave similar results (Figure S1). These findings were confirmed in respective Bland–Altman plots.

Spike recovery with HPLC, enzymatic, and colorimetric method in mouse and rat plasma

In addition the spike recovery with all three methods was calculated (Table 1). Again a significant overestimation by the colorimetric method relative to HPLC and enzymatic methods was noted in both species, whereas the results of the HPLC and enzymatic methods were comparable, though from the statistical point of view significant different.

Impact of hemolysis on the resulting creatinine concentrations, when using the colorimetric or the enzymatic method

For both mice and rats, independent creatinine measurements were performed on pooled plasma samples prepared six times with respective erythrocyte lysates (Table 2). For mice both methods resulted in an overestimation of the creatinine concentrations (up to 60%) in samples with pronounced hemolysis. In rats the results of the enzymatic method were virtually unaffected by the addition of the different lysates, whereas the results obtained with the colorimetric method revealed a persistent increase in concentration.

DISCUSSION

In this study, we compared three methods for determining plasma creatinine in mice and rats, namely colorimetric picric acid, enzymatic, and HPLC approaches. As expected, we noted a consistent overestimation for both species by the colorimetric method, an effect that was even increasing with higher creatinine values. The comparison between HPLC and the enzymatic method gave a good agreement over the whole data range. We could demonstrate that hemolysis, simulated

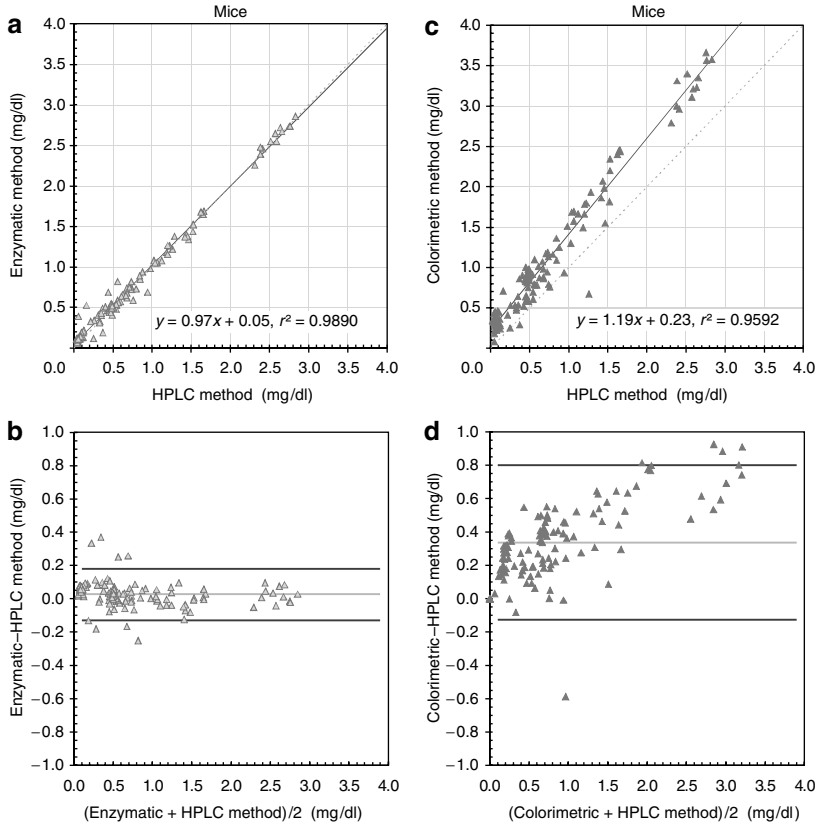

Figure 1 | Method comparison of creatinine measurement in mouse and rat plasma. Comparison of HPLC, enzymatic, and colorimetric method in (**a–d**) mice and (**e–h**) rats. (**a**, **c**, **e**, and **g**) scatter plots with regression line (solid), line of identity (broken), regression equation, and coefficient of determination (r^2) as well as (**b**, **d**, **f**, **h**) Bland–Altman plots for the comparison of creatinine concentrations in spiked (0.4 mg/dl creatinine added) and unspiked plasma of healthy as well as diseased mice ($n = 61$, plus 61 spiked samples) and rats ($n = 56$, plus 56 spiked samples) are depicted. Comparisons between the enzymatic method and HPLC (**a** and **b**) mice, (**e** and **f**) rats and the colorimetric method and HPLC (**c** and **d**) mice, (**g** and **h**) rats are shown in this figure, whereas the comparison between the enzymatic and colorimetric method is added as Supplementary Material. In the Bland–Altman plots the middle line indicates the mean difference; the outer lines indicate the limits of agreement (mean $\pm$ 2s.d.). Calibration curves for HPLC were for mice: $y = 0.0654x + 0.0187$, $r^2 = 0.9981$, and rats: $y = 0.0654x$–0.0335, $r^2 = 0.994$. Figure 1 continued on the following page.

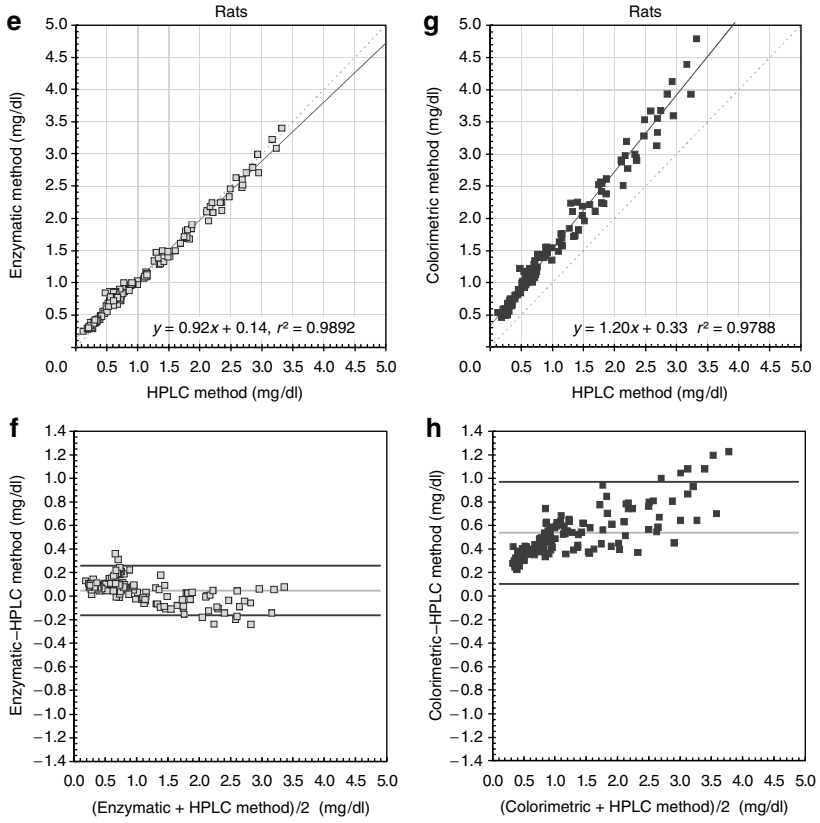

Figure 1 | Continued

Table 1 | Spike recovery in plasma of healthy and diseased mice and rats using the colorimetric, enzymatic, and HPLC method

Species/method	Endogenous min/max	Creatinine (mg/dl) Spike recovery[a] Median	P25	P75
Mice				
Colorimetric	0.08/3.35	0.60[b]	0.54	0.67
Enzymatic	0.06/2.72	0.43[b]	0.40	0.45
HPLC	0.02/2.66	0.46[b]	0.43	0.48
Rats				
Colorimetric	0.46/3.93	0.65[b]	0.56	0.69
Enzymatic	0.25/3.09	0.42[b]	0.40	0.45
HPLC	0.12/3.23	0.40[b]	0.32	0.43

[a]Spike recovery=spiked value – endogenous creatinine of an animal.
[b]Indicates a significant difference ($P<0.05$, nonparametric ANOVA) between all three methods.
For each animal (61 mice and 56 rats) endogenous creatinine and a spiked plasma sample (0.4 mg/dl creatinine spike) was measured with all three methods. The minimum (min) and maximum (max) of the endogenous creatinine, as well as the recovery of the spike (median, as well as 25th (P25) and 75th (P75) percentiles are used, as data were not normally distributed) are depicted.
ANOVA, analysis of variance; HPLC, high-performance liquid chromatography.

by adding hemoglobin, has a compromising effect on the results obtained with the enzymatic method in mice, but not in rats, when hemolysis itself is clearly visible. With the colorimetric method a strong hemoglobin interference was found for rats but not for mice.

The noted overestimation of plasma creatinine concentrations in mice and rats by the colorimetric method has been described previously and is a result of the interference of non-creatinine chromogens.[4,7,8] To overcome this imprecision, different HPLC methods for the measurement of plasma creatinine in mice have been established.[7,8] In our experiments, HPLC and enzymatic method gave comparable results. This is true despite the fact that in the spike experiment we noted statistically significant differences. This, however, is not relevant on the physiological level, as the differences in the respective medians are negligible. As the enzymatic method is much easier to perform, as no sample preparation is needed, our data reveal, that the enzymatic method represents a good alternative to HPLC measurements for routine screening tests. Furthermore our findings confirm that results obtained with the colorimetric method are difficult to interpret in mice and rats.

Hemoglobin has been considered as one of the interfering chromogens in the colorimetric method and in various enzymatic tests.[11] With the colorimetric measurement an interference of hemoglobin was observed and was more pronounced in rats than mice. With the enzymatic measurement an interference was noted in mice only when hemolysis became visible. Therefore special care must be taken during blood sampling, as the erythrocytes of mice and rats are much more sensitive to hemolysis than human erythrocytes.[12]

Table 2 | Interference of erythrocyte lysate with creatinine measurement

	Creatinine (mg/dl)			
	Mice		Rats	
Hemoglobin (mg/ml)	Colorimetric	Enzymatic	Colorimetric	Enzymatic
0	0.14±0.04	0.08±0.01	0.42±0.04	0.28±0.01
0.019	0.17±0.04	0.09±0.01	0.53±0.02[a]	0.28±0.01
0.039	0.14±0.04	0.08±0.01	0.53±0.03[a]	0.27±0.02
0.078	0.15±0.04	0.09±0.01	0.52±0.04[a]	0.27±0.01
0.156	0.18±0.03	0.10±0.02	0.52±0.02[a]	0.28±0.01
0.313	0.14±0.03	0.10±0.02	0.51±0.04[a]	0.26±0.01
0.625	0.15±0.03	0.10±0.01	0.54±0.03[a]	0.26±0.01
1.25[b]	0.19±0.02	0.12±0.01[a]	0.55±0.05[a]	0.26±0.02
2.5[b]	0.20±0.04	0.14±0.01[a]	0.58±0.02[a]	0.26±0.02
5[b]	0.23±0.02[a]	0.14±0.01[a]	0.49±0.05	0.23±0.01[a]

[a]Indicates a significant difference ($P<0.05$, parametric ANOVA) from the samples without hemoglobin addition.
[b]Hemolysis visible by eye.
Impact of the addition of lysate of erythrocytes on plasma creatinine concentrations determined with the colorimetric and enzymatic method. Lysate was quantified by its hemoglobin concentration. Given are mean value ±s.d. (n=6 per data set).

MATERIALS AND METHODS

Measurement of creatinine

Creatinine concentrations in plasma samples were measured with a rate-blanked and compensated picric acid colorimetric assay (CREA, No. 11489291216, Roche Diagnostics) based on the Jaffé method[1] and with an enzymatic assay (CREA plus, No. 11775642216, Roche Diagnostics, Mannheim, Germany) based on a cascade of reactions with the aid of creatininase, creatinase, and sarcosine. The finally liberated hydrogen peroxide is used to form a chromogen by oxidase. The color intensity is directly proportional to creatinine concentration.[3] Both assays were run in parallel on a Hitachi 911 Autoanalyzer (Roche Diagnostics).

For HPLC measurement plasma samples were stored at −20°C until analysis. After thawing plasma was centrifuged at 5000 r.p.m. for 6 min. Cold acetonitrile (100 μl), acidified with 1:200 (vol/vol) glacial acidic acid, was added to 25 μl of plasma, vortexed, and allowed to stand at 4°C for 15 min to precipitate plasma proteins. After centrifugation at 10 000 r.p.m. for 10 min in a refrigerated centrifuge, 100 μl of the supernatant was transferred to a tube and evaporated to dryness in a Univapo (UniEquip, Martinsried, Germany). The residue was resuspended in 25 μl of HPLC mobile phase (Biorad, Munich, Germany, No. 195-8513, available upon request) and vortexed for 20 s. After 10 min ultrasound treatment samples were centrifuged at 14 000 r.p.m. for 10 min and transferred to autosampler vials with low dead volume inserts (Roth, Karlsruhe, Germany, No. C 516.1, No. E 159.1). The vial was centrifuged again with 3000 r.p.m. for 5 min to ensure that any undissolved material remained at the bottom of the vial. The HPLC system employed was an Agilent 1100 Series (Waldbronn, Germany), HPLC column (cation exchange, weak) was purchased from Biorad (Munich, Germany, No. 195-5812, available upon request). Column oven temperature was 30°C, flow rate was set at 0.5 ml/min, and injection volume was 20 μl. Detection of eluting creatinine peak was achieved at 230 nm at about 4.4 min. The concentration of creatinine was calculated from a standard regression line based on pool plasma with creatinine spikes of 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/dl for mouse and 0.2, 0.4, 0.6, 0.8, and 1.0 mg/dl for rat plasma.

Animals and sample preparation

All samples were obtained from ongoing experiments, which were performed in accordance with Federal and Local Laws. Blood samples were taken from the retrobulbar sinus of mice and rats, under isoflurane anesthesia and placed in heparin tubes (Sarstedt, Nümbrecht, Germany).

For the comparison of all three measurements, blood was collected from SD, PKD/Mhm, and PKD2mutL247 rats and pcy, Bl6, FGF-2, and CD1 mice. SD rats as well as Bl6, FGF-2, and CD1 mice are models for healthy animals, PKD/Mhm, and PKD2mutL247 rats[13] and pcy mice are models of polycystic kidney disease. Samples were additionally spiked with 10 μl of a creatinine standard stock solution in 0.2N HCl (Sigma, Munich, Germany) or 10 μl 0.2N HCl to controls.

In the hemoglobin experiment plasma of SD rats and Bl6 mice were used. Erythrocytes from the pooled blood samples were washed three times with 0.9% NaCl and lysed in three freeze-and-thaw cycles and ultrasound treatment. Free hemoglobin was measured and mouse or rat plasma was spiked with 0.02–5 mg/ml hemoglobin. Each sample was prepared six times independently and measured with the enzymatic and the colorimetric method.

Statistics

For the statistical evaluation the SAS (Version 9.1,. SAS Institute, Cary, NC, USA) procedures PROC NPAR1WAY (non-parametric analysis of variance), PROC GLM (parametric analysis of variance), PROC GPLOT (graphs), PROC MEANS (means, s.d.), PROC REG (regression analysis), and PROC UNIVARIATE (test for normal distribution) were used. All normally distributed data are expressed as mean value±s.d., whereas for non-normally distributed data median and percentiles (25th and 75th) are given. For a direct comparison of the different methods a regression analysis and the Bland–Altman[14] approach was chosen. In the analysis of variance a P-value <0.05 was considered significant.

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SUPPLEMANTARY MATERIAL

Figure S1. Comparison of the enzymatic and colorimetric creatinine measurement in mice and rats.

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