http://www.kidney-international.org

Tandem mass spectrometry measurements of creatinine in mouse plasma and urine for determining glomerular filtration rate

N Takahashi[1,2,4], G Boysen[3,4], F Li[1], Y Li[3] and JA Swenberg[3]

[1]*Department of Pathology and Laboratory Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA;* [2]*Department of Cell and Molecular Physiology, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA and* [3]*Department of Environmental Sciences and Engineering, Center of Environmental Health and Susceptibility, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

Endogenous creatinine clearance (Ccr) is widely accepted as an estimate of glomerular filtration rate (GFR), the best overall biomarker of kidney function. However, current common methods of measuring creatinine are not sensitive enough for mouse plasma. Accordingly, we here report a new method of measuring creatinine by liquid chromatography tandem mass spectrometry (LC-MS/MS) using deuterated [$^2H_3$]-creatinine as an internal standard. The assay requires 10 μl or less of plasma or urine, and is eight times more sensitive than high-performance liquid chromatography. The reproducibility of the assay of replicates is approximately $\pm$10%. The plasma creatinine levels of wild type male C57BL/6J mice obtained by LC-MS/MS are 0.076$\pm$0.002 mg/dl (n = 65). To estimate daily urinary creatinine excretion for calculating Ccr, we collected urine from mice housed in metabolic cages, and combined this with washes from the cage internal surfaces. Creatinine in the wash varies from 4 to 67% of the total daily urinary creatinine excretion (typically $\sim$400 μg/day). Ccr obtained by LC-MS/MS was 329$\pm$17 μl/min, which is indistinguishable from GFR measured by using fluorescein isothiocyanate-inulin. The LC-MS/MS method is sensitive, specific, simple, fast, and inexpensive; it is suitable for estimating GFR in conscious mice or other small animals. As it allows repeated measurements in the same animals, it facilitates detection of subtle differences or changes in renal function.

Kidney International (2007) **71,** 266–271. doi:10.1038/sj.ki.5002033; published online 6 December 2006

KEYWORDS: creatinine; GFR; mice; mass spectrometry; LC-ESI-MS/MS; plasma; urine

Correspondence: *N Takahashi, The University of North Carolina at Chapel Hill, 518 Brinkhous-Bullitt Building, Chapel Hill, North Carolina 27599-7525 USA. E-mail: ntakaha@med.unc.edu*

[4]These authors contributed equally to this work

Received 7 August 2006; revised 17 October 2006; accepted 24 October 2006; published online 6 December 2006

Glomerular filtration rate (GFR) is the best overall biomarker of kidney function. The gold standard for GFR determination depends on measuring the clearance of a small molecule such as inulin that is completely filtered in the glomeruli, is not reabsorbed or secreted by renal tubules, and is not metabolized or secreted anywhere else in the body. However, the use of fluorescein isothiocyanate (FITC)-inulin could trigger the generation of antibodies against it in mice, which makes the use of FITC-inulin not suitable for measuring GFR repeatedly over the period of months in the same animals. Creatinine clearance (Ccr) is an acceptable alternative. However, current common methods of measuring creatinine are not sufficiently sensitive enough for use with mouse plasma.[1–3] Consequently, although more and more mouse models of human diseases have been generated by gene targeting and transgenic techniques, the lack of a simple and reliable method to accurately measure creatinine in mouse plasma and urine has limited the identification of genetic and environmental factors that affect kidney function. The most prevalent methods to measure creatinine in human samples are the alkaline picrate method using Jaffé's reaction,[4] and the enzymatic method using creatinine amidohydrolase, creatine amidinohydrolase, sarcosine oxidase, and peroxidase, modified from Moss *et al.*[5] High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is an alternative method for measuring plasma creatinine, but proteins in the samples need to be eliminated before applying samples to the HPLC column,[6] which introduces errors due to various recovery rates. Moreover, if molecules that have the same retention time as creatinine exist in the samples, HPLC may not be specific for creatinine. The use of liquid chromatography combined with mass spectrometry has been reported to measure creatinine in human plasma,[7] but mouse plasma often gives a lot of background with this method (our unpublished observations). We have therefore developed a new method of measuring creatinine using an internal standard labeled with a non-radioactive stable isotope combined with liquid chromatography, electrospray ionization, and tandem mass spectrometry (LC-MS/MS) to

increase the specificity and reduce background. This method is sensitive, specific, simple, fast, inexpensive, requires very small amount of body fluid, and allows repeated measurements in the same animals.

RESULTS

Specificity, reproducibility, and sensitivity of creatinine measurement using LC-MS/MS

In developing our new procedure we determined the limit of detection and reproducibility of measuring creatinine by LC-MS/MS. First, synthetic standard solutions of creatinine and creatinine-*N*-methyl-D3 ($[^2H_3]$-creatinine) were quantified by UV spectrometry using the published absorption coefficient ($\xi_{0\ \lambda=240}$) of 6900.[8] Variability of UV measurement was $\pm$7%. These solutions were used to prepare calibration solutions. The calibration solutions, containing different amounts of creatinine and a constant amount (2950 pg) of $[^2H_3]$-creatinine, were used for instrument calibration and quality control during sample analysis. Creatinine and $[^2H_3]$-creatinine both have the same retention time of approximately 2.2 min, but their mass-to-charge ratios (m/z) when ionized differ, being 114 and 117, respectively. When creatinine is fragmented, the main fragment observed has an m/z of 44. When $[^2H_3]$-creatinine is fragmented, the corresponding peak has an m/z of 47. The LC-MS/MS method combines these features by examining HPLC peaks that have retention time of 2.2 min, and tandem mass spectrometer fragments with m/z transition of 114 to 44 for creatinine, or 117 to 47 for $[^2H_3]$-creatinine. This combination makes the LC-MS/MS method more specific than either HPLC or liquid chromatography combined with mass spectrometry. Thus no $[^2H_3]$-creatinine peak was detected in our creatinine standard, and no creatinine peak was detected in our $[^2H_3]$-creatinine standard by LC-MS/MS, indicating no cross-talk between the two materials.

The instrument outputs data in the form of (area of creatinine peak) which has a retention time of 2.2 min and an m/z transition of 114 to 44 $\div$ (area of $[^2H_3]$-creatinine peak) which has a retention time of 2.2 min and an m/z transition of 117 to 47, and we found this ratio to be linear with a regression coefficient of 0.995 over a range of 17–1720 pg of creatinine/injection, which covers the range likely from real samples (Figure 1). The amount of creatinine in the sample is calculated as the ratio of (area of creatinine peak) $\div$ (area of $[^2H_3]$-creatinine peak) $\times$ (the amount of $[^2H_3]$-creatinine initially added to the sample) $\times$ (a response factor). The response factor (1.16) corrects the difference in ionization efficiency of creatinine and $[^2H_3]$-creatinine, and the response of the detector to them. It was determined with the calibration solution as (creatinine concentration $\times$ area of $[^2H_3]$-creatinine peak) $\div$ ($[^2H_3]$-creatinine concentration $\times$ area of creatinine peak). The reproducibility for analyses of urine and plasma was $\pm$11% (standard deviation) and $\pm$15%, respectively, as determined by six repetitive analyses of one urine and one plasma sample. The limit of detection was 30 pg/5 μl injection, with a signal to noise ratio greater than 10. In effect this means that the method can be used to measure creatinine concentrations as low as 0.013 mg/dl of creatinine. This represents approximately eightfold higher sensitivity compared to previously published HPLC-UV method.[6]

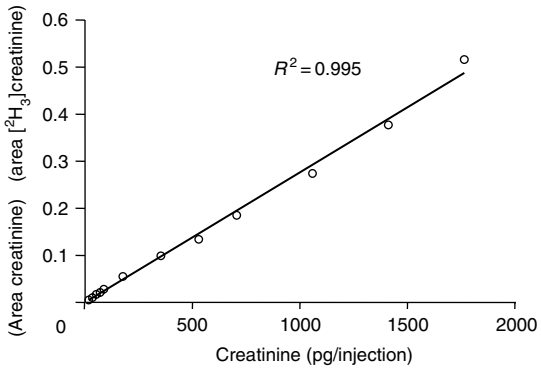

Figure 1 | Calibration curve for creatinine. Constant amount (2950 pg) of $[^2H_3]$-creatinine mixed with various amounts (17–1720 pg) of creatinine were tested by LC-MS/MS. The ratios of (area of creatinine) $\div$ (area of $[^2H_3]$-creatinine) by LC-MS/MS are plotted against the amount of creatinine injected.

Measuring mouse urine creatinine using LC-MS/MS

For method comparison, we next determined urinary creatinine concentrations in several strains of mice by LC-MS/MS, Jaffé's alkaline picrate method, and the enzymatic method. Representative ion chromatograms of creatinine from a mouse urine sample are shown in Figure 2. In the upper panel the only peak observed with a transition of 114 to 44 is creatinine eluting at 2.2 min. In the lower panel the only peak observed with a transition of 117 to 47 is $[^2H_3]$-creatinine eluting at 2.2 min. (The signal at 1 min is caused by switching the effluent flow from waste to the LC-MS/MS, and does not represent a real compound.) The creatinine concentration in this sample is calculated as shown in the legend of Figure 2.

Figure 3a–e compares the creatinine concentrations measured by three methods. Values obtained by Jaffé's method were 20% higher than those measured by LC-MS/MS (Figure 3d, $P<0.0001$). The enzymatic method also gave higher values than LC-MS/MS, especially when urine volumes were larger, and when urine creatinine concentrations were lower (Figure 3c). The enzymatic method gave more than 10 times higher values in some samples than LC-MS/MS, revealing major differences (Figure 3e).

Measuring murine plasma creatinine using LC-MS/MS

It has been difficult to determine plasma creatinine concentrations in mice, because they are approximately 100 times lower than those of urine, and because the current common methods of creatinine measurement lack sufficient sensitivity for measuring mouse plasma creatinine in the small volume of plasma available. For an accurate determination of GFR in mice, one goal is to establish a highly sensitive

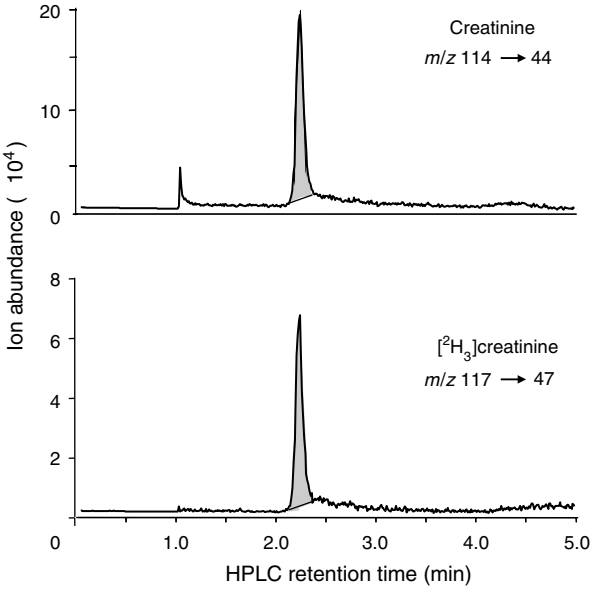

Figure 2 | Ion chromatograms of mouse urine creatinine measurement using LC-MS/MS. The sample was prepared as described in Materials and Methods. The upper and lower panels show ion transition of *m/z* (mass-to-charge ratio) from 114 to 44 and from 117 to 47 for creatinine and [$^2H_3$]-creatinine, respectively, both having the same retention time (2.2 min). The areas under the curves of creatinine and [$^2H_3$]-creatinine shown in gray are 788800 and 275166, respectively. The creatinine concentration of this urine is $788800 \div 275166 \times 0.96$ ng/μl (concentration of internal standard) $\times$ 1.16 (response factor) $\times$ 100 (dilution factor) = 319 ng/μl = 31.9 mg/dl.

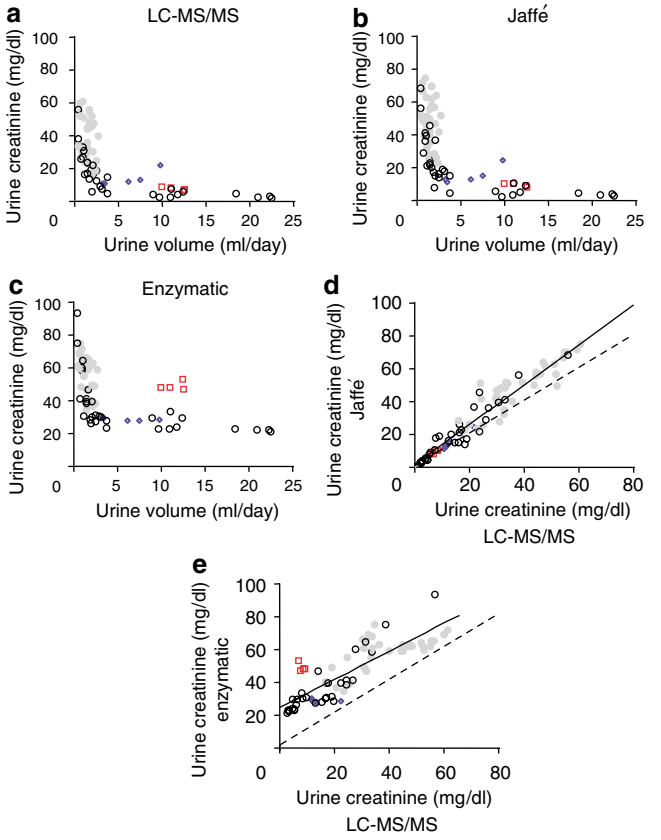

Figure 3 | Comparison of mouse urine creatinine measured by LC-MS/MS, Jaffé's and enzymatic methods. Mouse urine creatinine measured by (**a**) LC-MS/MS, (**b**) Jaffé's alkaline picrate method, and (**c**) the enzymatic method plotted against daily urine volume. (**d** and **e**) Urine creatinine concentrations measured by Jaffé's method and the enzymatic method plotted against those measured by LC-MS/MS. Closed gray circle: wild-type controls ($n = 32$); open black circle: diabetic mice ($n = 39$); red square: furosemide-sensitive kidney-specific NaK2Cl cotransporter NKCC2 (*Slc12a1*)-deficient mice ($n = 4$);[12] blue diamond: *Ren1c*-deficient mice ($n = 6$).[16] Solid lines show linear fit lines. **d:** $y = 1.85 + 1.21x$, $R^2 = 0.92$. **e:** $y = 25.1 + 0.8x$, $R^2 = 0.71$. Dashed lines show lines of equivalence.

method for quantitation of plasma creatinine. Therefore, plasma samples were analyzed from mice using LC-MS/MS. Creatinine was easily detected in all plasma specimens and representative ion chromatograms are shown in Figure 4. The plasma creatinine values of wild-type male mice (3–6 months of age) measured by LC-MS/MS were 0.076 ± 0.002 mg/dl ($n = 65$), whereas the mean plasma creatinine values measured by HPLC in the literature are 0.10–0.22 mg/dl,[6,9,10] suggesting that HPLC overestimates the concentration of creatinine in mouse plasma.

Mouse Ccr

Using the urine and plasma creatinine values, Ccr (μl/min) is calculated as total daily urinary creatinine excretion (μg/day) $\div$ plasma creatinine concentration (μg/μl) $\div$ 1440 (to convert day to min). However, with the animals as small as mice it is difficult to obtain accurate estimates of daily urinary creatinine excretion because in metabolic cages with the inner diameter of 15 or 20 cm, there is often no urine in the urine collection tube with normal adult mice, showing that significant amounts of urine have either evaporated to dryness or been lost. To overcome this difficulty, we reduced the inner diameter of the domicile portion of the metabolic cages to 10 cm, which reduces the fraction of urinary creatinine retained on the cage internal surfaces. More importantly, we rinsed the internal surfaces of the cages with 20 ml of water, measured the creatinine content of the rinse by LC-MS/MS, and added this to the amount of creatinine in the urine collection tubes to obtain an accurate excretion of total daily urinary creatinine. (Jaffé's method is not sensitive enough to measure creatinine in the wash, which makes LC-MS/MS superior to Jaffé's method in accurate determination of urinary creatinine excretion.) The importance of this is illustrated by noting that the amount of creatinine remaining on the cages varies from 4 to 67% (median 6%, mean 11%) of the total daily urinary creatinine excretion.

If food was present in the urine collection tubes, or the funnels of the cages were clogged with food, samples were excluded. In these cases an accurate measurement of daily urinary creatinine excretion is not possible as 1 g of normal chow contains 160 μg creatinine measured by LC-MS/MS.

Using this LC-MS/MS method, the Ccr of 6-month-old male wild-type C57BL/6J mice in this study was 329 ± 17 μl/min/mouse (Table 1). When 24 h Ccr and FITC-inulin

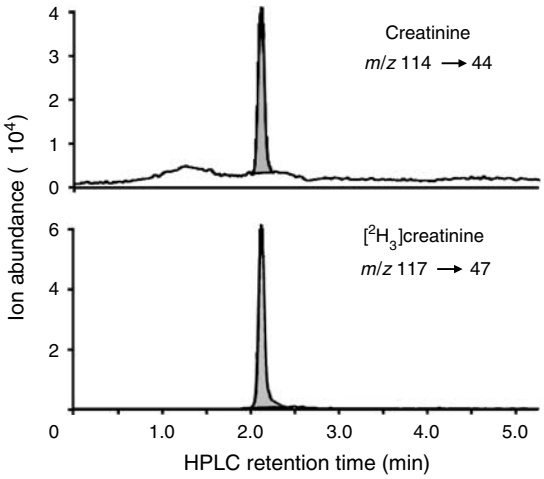

Figure 4 | Ion chromatograms of mouse plasma creatinine measurement using LC-MS/MS. The sample was prepared as described in Materials and Methods. The top and bottom panels show ion transition of *m/z* from 114 to 44 and from 117 to 47 for creatinine and [$^2H_3$]-creatinine, respectively. Creatinine and [$^2H_3$]-creatinine were retained for 2.2 min, and the areas under the curve shown in gray are 118860 and 223286, respectively. Creatinine concentration of this plasma is 118860 ÷ 223286 × 0.96 ng/μl (concentration of internal standard) × 1.16 (response factor) = 0.59 ng/μl = 0.059 mg/dl.

clearance were measured in the same animals simultaneously, Ccr measured by LC-MS/MS was 311 ± 11 μl/min and FITC-inulin clearance was 291 ± 24 μl/min ($n = 7$). The difference between them was 20 ± 17 μl/min, which was not different from zero ($P = 0.28$). There was a significant correlation between the two methods ($R^2 = 0.58$, $P = 0.04$). These results demonstrate that tubular secretion of creatinine is minimal, and that 24-h Ccr measured by LC-MS/MS is suitable for estimating 24-h GFR.

Ccr was also examined in age-matched male C57BL/6J mice that have been diabetic for 3 months after the onset of streptozotocin-induced hyperglycemia (Table 1). Diabetic mice tended to eat and urinate more, but their plasma creatinine concentration, daily urinary creatinine excretion, and Ccr were similar to those of non-diabetic control mice. In contrast, mice lacking *Ren1c* weighed less, urinated more, and showed higher plasma creatinine concentration and lower Ccr compared to those of control mice, suggesting that GFR of the *Ren1c*−/− mice was decreased.

DISCUSSION

Accurate and specific quantitation of plasma creatinine in mice has been a problem in estimating GFR. We report here the suitability of LC-MS/MS for quantitation of creatinine in small (10 μl) volumes of mouse plasma and urine to calculate Ccr. Our method of LC-MS/MS has distinct advantages over others. As the LC-MS/MS includes an internal standard, not present in other methods, this greatly increases the accuracy of our method. In addition, because LC-MS/MS selects peaks with *m/z* transition of 114 to 44 for creatinine and 117 to 47 for [$^2H_3$]-creatinine that have retention time of 2.2 min, LC-MS/MS significantly increases specificity and reduces background noise (and hence limit of detection) compared to HPLC or liquid chromatography combined with mass spectrometry. LC-MS/MS is approximately eight times more sensitive than HPLC-UV. The most common methods for creatinine measurements, Jaffé's alkaline picrate and the enzymatic methods lack sufficient sensitivity for analysis of plasma creatinine in mice.

Creatinine concentration in the urine measured by the LC-MS/MS method was lower compared to the enzymatic and Jaffé's methods. The enzymatic method depends on first converting creatinine to creatine; it consequently does not discriminate creatinine from creatine.[5] As mouse urine contains an equal or higher amount of creatine compared to creatinine,[11] the enzymatic method is not suitable for urine creatinine analysis in mice. The error becomes especially large when the urine volumes are larger than ~8 ml/day, such as in diabetic mice and in furosemide-sensitive NaK2Cl-deficient mice,[12] probably because at high urine volumes absorption of creatine in tubular fluid is less complete.[13]

The Ccr value obtained by HPLC (255 μl/min/mouse)[6] is lower than our values, which is probably because HPLC overestimates the concentration of creatinine in plasma more than in urine, as plasma contains non-creatinine substances that have the retention time indistinguishable from that of creatinine. The abundance of these substances could reach ~60% of that of creatinine (our unpublished observation by liquid chromatography combined with mass spectrometry). With essentially no sample workup and relative fast analysis time, the LC-MS/MS method is applicable to studies with large sample numbers that require fast turn around (high throughput). Currently the analysis takes less than 8 min per sample and future improvements using ultra high pressure LC-MS/MS can potentially reduce the analysis time by a

Table 1 | Renal function of 6-month-old male C57BL/6J mice

	Body weight (g)	Food intake (g)	Plasma glucose (mg/dl)	Urine volume (ml/day)	Plasma creatinine (mg/dl)	Urine creatinine (μg/day)	Creatinine clearance (μl/min)
Control (*n*=10)	33.2 ± 1.2	4.7 ± 0.2	144 ± 18	1.5 ± 0.2	0.089 ± 0.005	417 ± 45	329 ± 17
Diabetes (*n*=13)	31.0 ± 1.1	5.8 ± 0.4	393 ± 26**	3.8 ± 1.6	0.101 ± 0.009	474 ± 52	350 ± 44
Ren1c−/− (*n*=6)	25.4 ± 1.4*	5.7 ± 0.4	ND	6.6 ± 0.8*	0.207 ± 0.021*	477 ± 31	167 ± 17*

ND, not determined.
Diabetic mice were made diabetic using streptozotocin and have been diabetic for 3 months. Plasma and urine creatinine was measured by LC-MS/MS.
*$P < 0.05$ and **$P < 0.001$.

factor of 5–10,[14] which will make the analysis even faster. The costs of LC-MS/MS and of the enzymatic method are similar, and the availability of mass analyzers is rapidly increasing.

A major challenge of measuring Ccr using metabolic cages with small animals is to completely recover urinary creatinine. Our metabolic cages are designed to minimize the amount of creatinine retained in the cages as a result of evaporation. We demonstrate a simple correction for this problem, easily executed in combination with the LC-MS/MS method, namely the need to rinse the cages and estimate creatinine in the rinse fluid. Amount of creatinine remaining on the metabolic cages varied from 4 to 67% of total daily urinary creatinine excretion, indicating the importance of rinsing cage internal surfaces to completely recover urinary creatinine. Food contamination was minimal in our experiments, but to minimize contamination of creatinine from the food the use of vegetarian diets should be helpful. Indeed, creatinine in our regular chow is 160 μg/g, whereas in vegetarian diets it is less than 2 μg/g in three diets that we tested using LC-MS/MS.

In conclusion, LC-MS/MS enables us to repeatedly measure plasma and urine creatinine and estimate GFR in conscious mice and other small animals without the complications of significant blood loss and difficulties owing to retention of urine in the metabolic cages. LC-MS/MS is expected to be sensitive enough to monitor mild progressive exacerbation of kidney function such as in diabetic nephropathy. It also makes it much easier to calculate fractional excretions of electrolytes and other substances. LC-MS/MS is simple, sensitive, specific, inexpensive, and requires no radioactive materials. It should therefore be applicable for a wide variety of species, for many types of investigation where estimation of kidney function are required.

MATERIALS AND METHODS

Materials

Creatinine was from Sigma (St Louis, MO, USA), and creatinine-*N*-methyl-D3 ([$^2H_3$]-creatinine) was from Cambridge Isotope Laboratory (Cambridge, MA, USA). All other solvents and reagents were from Fisher and American Chemical Society grade or higher.

General

All animal experiments were conducted in accordance with the guidelines of IACUC at UNC. Some mice were made diabetic by intraperitoneally injecting streptozotocin (40 mg/kg/day for 5 consecutive days) or vehicle (citrate buffer) as described previously,[15] and were observed daily for their general health. Mice were considered diabetic if blood glucose level exceeded 300 mg/dl. All animals were fed Laboratory Rodent Diet 5001 (Lab Diet, Richmond, IN, USA).

Kidney function tests

Individual mice were housed in metabolic cages designed for mouse renal function studies.[12,16] Body weight, the amount of food and water intakes, and urine volume in 24 h were monitored for 2 consecutive days (48 h). Wire mesh and the bottom of collection vessel of the cages were washed with 20 ml water every 24 h to recover the urine adhering to the cages. Urine samples of the latter 24 h were used to measure urine creatinine. Blood was drawn to measure plasma creatinine at the end of the 48 h to avoid perturbing physiological steady state GFR.[16] Because mice are inactive and their food intake is minimal in the daytime, the plasma creatinine in the afternoon is considered to be least affected by muscle activity and food-derived creatinine. Therefore, setting up cages and collecting samples were carried out between 15:00 and 16:00. It is important to set up metabolic cages and draw blood at the same time for all animals to eliminate the effect of circadian variation of plasma creatinine. Plasma and urine samples were kept at -20°C until analysis. Many of the previously collected urine samples were also used for method comparison. For comparison to the novel LC-MS/MS method urinary creatinine was also determined by the enzymatic method using a VT250 Chemical Analyzer (Johnson & Johnson, New Brunswick, NJ, USA),[16] and Jaffé's alkaline picrate method using a kit (Exocell Inc., Philadelphia, PA, USA). For these methods urine samples were diluted depending on the urine volume, normally 1:20, and 10 μl was used for analysis. To compare 24 h Ccr and FITC-inulin clearance, one 14-day-release 50 mg-FITC-inulin pellet (Innovative Research of America, Sarasota, FL, USA) was implanted subcutaneously to 3 months old C57BL/6J males. Six days later animals were housed in metabolic cages to obtain 24 h urine and plasma as described above, and simultaneous Ccr and inulin clearance in the same animals were calculated as described previously.[2] In this experiment, the urine collection tube was covered with aluminum foil to avoid exposure to the light. Ten microliter of plasma, 1:100 diluted urine, or of cage wash was mixed with 10 μl of 500 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid pH 7.4, and 30 μl phosphate-buffered saline, and FITC fluorescence was determined with 485 nm excitation and 538 nm emission. The linear range of the standard curve of FITC intensity against FITC-inulin concentration is much narrower than that of creatinine with LC-MS/MS, because the slope of the standard curve becomes less steep when FITC concentrations are high. Therefore, it is important to dilute urine before measurement to avoid underestimating the values.

Preparation of samples

For accurate quantitation, 9.6 ng (10 μl of 0.96 ng/μl) [$^2H_3$]-creatinine and either 10 μl of urine that had been diluted with water (1:100), 10 μl of the cage wash, or 10 μl plasma were added to 200 μl of absolute ethanol and vortexed, followed by centrifugation at 12,000 r.p.m. in a microfuge for 15 min at 4°C. The protein-free supernatant was transferred to a new tube taking care not to remove any particular matter, and solvent was removed under vacuum. Plasma samples were then dissolved in 200 μl of water, extracted twice with one volume chloroform, and 5 μl were analyzed by LC-MS/MS. Note that the internal standard was added before the protein precipitation to correct inconsistent recovery during the ethanol precipitation, which ranged from 60 to 90% (mean 80.1%) calculated from the amounts of internal standard recovered. Deproteinized and evaporated urine samples or cage washes were dissolved in 200 μl water and 5 μl were analyzed directly by LC-MS/MS without chloroform extraction. To quantify creatinine in the food, 5 g of food was mixed in 20 ml of water, centrifuged, and 10 μl of the supernatant was deproteinized and delipidated as described above before the analysis by LC-MS/MS. The diets tested were regular chow Lab Diet 5001 and vegetarian diets: 2016, TD.94045, and TD.96329 (Harlan Teklad, Madison, WI, USA).

Quantitative LC-MS/MS analyses of creatinine

The quantitative analysis of the creatinine by LC-MS/MS was performed with a Surveyor LC coupled to a TSQ-Quantum Classic triple quad mass analyzer (ThermoFinnigan, San Jose, CA, USA). A 2.0×150 mm YMC C_{18}, 5 μm column was operated with a linear gradient of 5% acetonitril 0.1% formic acid for 0.3 min, then to 95% acetonitril 0.1% formic acid in 1 min at a flow rate of 200 μl/min. During the first minute the effluent flow is diverted to waste to prevent salts from entering the MS. The creatinine retention times were determined with authentic standards. Creatinine and $[^2H_3]$-creatinine were detected in single reaction monitoring mode, monitoring the transitions of the m/z 114 to 44 and m/z 117 to 47, respectively. The MS and electro spay ionization parameters were optimized by direct infusion of standard using the Xcalibur software (ThermoFinnigan, San Jose, CA, USA). The conditions were as follows: spray voltage 2200 V, heated capillary temperature 350°C.

Statistical analyses

Values are expressed as mean $\pm$ s.e.m. unless mentioned. Data were analyzed by linear fit, Student's t-test or analysis of variance with Tukey–Klamer *post hoc* test using JMP software version 6 (SAS Institute Inc., Cary, NC, USA).

ACKNOWLEDGMENTS

We thank Drs Oliver Smithies, Chuck Benton, Mr Longquan Xu, and Mr Leonard B Collins for their assistance. This work is supported by Grants from the National Institutes of Health (U01HL070523, HL49277, P30 ES10126) and American Heart Association (0265464U).

REFERENCES

1. Meneton P, Ichikawa I, Inagami T *et al.* Renal physiology of the mouse. *Am J Physiol Renal Physiol* 2000; **278**: F339–F351.
2. Qi Z, Whitt I, Mehta A *et al.* Serial determination of glomerular filtration rate in conscious mice using FITC-inulin clearance. *Am J Physiol Renal Physiol* 2004; **286**: F590–F596.
3. Yun JC, Delea CS, Bartter FC *et al.* Increase in renin release after sinoaortic denervation and cervical vagotomy in anesthetized dogs (II) (39903). *Proc Soc Exp Biol Med* 1977; **156**: 186–191.
4. Bonsnes RW, Taussky HH. On the colorimetric determination of creatinine by Jaffé reaction. *J Biol Chem* 1945; **158**: 581–591.
5. Moss GA, Bondar RJ, Buzzelli DM. Kinetic enzymatic method for determining serum creatinine. *Clin Chem* 1975; **21**: 1422–1426.
6. Dunn SR, Qi Z, Bottinger EP *et al.* Utility of endogenous creatinine clearance as a measure of renal function in mice. *Kidney Int* 2004; **65**: 1959–1967.
7. Stokes P, O'Connor G. Development of a liquid chromatography-mass spectrometry method for the high-accuracy determination of creatinine in serum. *J Chromatogr B Analyt Technol Biomed Life Sci* 2003; **794**: 125–136.
8. Dawson R, Elliott D, Elliott W *et al. Data for Biomedical Research*, 3rd edn, Gloucestershire (ed). Clarendon Press: UK, 1987.
9. Meyer MH, Meyer Jr RA, Gray RW *et al.* Picric acid methods greatly overestimate serum creatinine in mice: more accurate results with high-performance liquid chromatography. *Anal Biochem* 1985; **144**: 285–290.
10. Yuen PS, Dunn SR, Miyaji T *et al.* A simplified method for HPLC determination of creatinine in mouse serum. *Am J Physiol Renal Physiol* 2004; **286**: F1116–F1119.
11. Bernstein SE. Physiological Characteristics. In: *Biology of the Laboratory Mouse*. Green EL (ed). Dover Publications Inc.: New York, 1975.
12. Takahashi N, Chernavvsky DR, Gomez RA *et al.* Uncompensated polyuria in a mouse model of Bartter's syndrome. *Proc Natl Acad Sci USA* 2000; **97**: 5434–5439.
13. Walker JB. Creatine: biosynthesis, regulation, and function. *Adv Enzymol Relat Areas Mol Biol* 1979; **50**: 177–242.
14. Yu K, Little D, Plumb R *et al.* High-throughput quantification for a drug mixture in rat plasma-a comparison of Ultra Performance liquid chromatography/tandem mass spectrometry with high-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Spectrom* 2006; **20**: 544–552.
15. Gurley SB, Clare SE, Snow KP *et al.* Impact of genetic background on nephropathy in diabetic mice. *Am J Physiol Renal Physiol* 2006; **290**: F214–F222.
16. Takahashi N, Sequeira Lopez MLS, Cowhig CE *et al.* Ren1c homozygous null mice are hypotensive and polyuric, but heterozygotes are indistinguishable from wild type. *J Am Soc Nephrol* 2005; **16**: 125–132.