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A method for accurate measurement of GFR in conscious, spontaneously voiding rats

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A method for accurate measurement of GFR in conscious, spontaneously voiding rats. Renal function measurement by clearance methods relies on accurately timed urine collection. In small experimental animals, renal function measurement is usually performed under anesthesia and/or with the application of bladder catheters to ensure accurate urine collection. To avoid both anesthesia and the need for bladder catheters we developed a method to measure glomerular filtration rate (GFR) in spontaneously voiding conscious rats. GFR was measured as the urinary clearance of constantly infused ^{125}I-iothalamate. To correct for incomplete bladder emptying, urinary clearance of ^{125}I-iothalamate was multiplied by the ratio of plasma and urinary clearance of simultaneously infused ^{131}I-hippuran, a correction method that has been previously validated in humans. Reproducibility of the technique was evaluated by analysis of the results of four consecutive clearance periods during the day (intra-assay variation) in a group of 17 rats and of two consecutive clearance periods on two or three separate days in a group of 20 rats (inter-assay variation), all with normal renal function. Application of the correction method reduced the intra-assay coefficient of variation (mean ± SD) from 37.4 ± 14.3 to 5.4 ± 2.3% ($P < 0.05$). The mean inter-assay coefficient of variation fell slightly from 23.4 ± 10.3 to 11.0 ± 7.2% ($P < 0.10$). In rats with moderately impaired renal function ($N = 8$) the intra-assay variation fell from 27.9 ± 20.7 to 2.7 ± 1.6% ($P < 0.05$). Our data show that this correction method is a useful technique to assess renal function in conscious, spontaneously voiding rats.

Renal function measurement in small experimental animals is often performed by clearance methods. Since accurate measurement of the glomerular filtration rate (GFR) by clearance methods relies on well timed urine collections, most studies are carried out under anesthesia, or, if conscious, a few hours after instrumentation with bladder or ureter catheters. However, it is well-recognized that both anesthesia and surgery affect GFR as well as urinary excretion rates of sodium and water [1–3], even until several hours after anesthesia and instrumentation [1, 4].

To avoid the effects of anesthesia, several techniques have been developed to measure GFR in conscious rats [5–9]. As rats do not empty their bladder completely when voiding spontaneously [10], chronically implanted bladder catheters are applied by several investigators [5, 8] to ensure complete urine collection. Chronic instrumentation of the bladder in rats, however, is cumbersome and can induce urological complications [11, 12]. In the present study, therefore, we present a method for accurate measurement of renal function as the clearance of constantly intravenously infused ^{125}I-iothalamate in conscious, spontaneously voiding rats. The problem of incomplete bladder emptying is circumvented by a correction method for incomplete urine collection. This correction method has been extensively validated in humans [13, 14]. The aim of the present study was to test its feasibility in rats with normal and moderately impaired renal function. In addition, we tested whether the correction method could also be applied when the tracer was administered by an intraperitoneal infusion route.

METHODS

Male Wistar rats 3 to 6 months of age, were studied (280 to 450 g). The animals were housed in individual cages in a temperature controlled room with a 12 hour light/dark cycle. All animals were fed with commercially available rat chow and tap water *ad libitum*. During the renal function experiments food and water were withheld to avoid the influence of food and water intake on renal function.

One week before the experiments the rats ($N = 17$) were instrumented. Anesthesia was induced with 4% isoflurane and N_2O:O_2 (1:2). The right jugular vein was catheterized using silicone tubing and the left carotid artery was catheterized with polyethylene tubing fused to a silicone tubing. The proximal end of each catheter was tunneled subcutaneously and exteriorized on the head. The catheter was fixed on the skull by a (modified) method previously described by Steffens [15]. The catheters were filled with a 60% polyvinylpyrrolidone solution in saline with 500 IU/ml heparin closed with a piece of heat-sealed polyethylene tubing.

In a second group of rats ($N = 8$) the right jugular vein was catheterized as described above and a silicone catheter was implanted in the peritoneal cavity through the abdominal wall. Both catheters were tunneled subcutaneously and fixed on the skull.

In a third group of rats ($N = 8$) moderate renal function impairment was induced by adriamycin. To this purpose adriamycin (1.5 mg/kg body wt; Pharmachemie B.V., Haarlem, Holland) was injected intravenously. This model is characterized by proteinuria and normal renal function at onset and, followed by gradual impairment of renal function late in the disease [16].

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Key words: renal function, conscious rats, spontaneous voiding.

Table 1. Time course of duration between voidings (Tc collection), urine voiding volume (Uvv), Urine flow (Uv) and GFR (mean ± SD; $N = 17$)

Period	Tc *min*	Uvv *ml*	Uv *ml/hr*	$GFR_{standard}$ *ml/min 100 g body wt*	$GFR_{corrected}$ *ml/min 100 g body wt*
1	60 ± 25	2.34 ± 0.88	2.48 ± 0.84	1.04 ± 0.33	0.99 ± 0.20
2	46 ± 11	1.86 ± 0.60	2.44 ± 0.77	0.82 ± 0.27	1.00 ± 0.22
3	60 ± 15	2.44 ± 0.74	2.47 ± 0.65	0.91 ± 0.21	1.02 ± 0.23
4	48 ± 11	1.89 ± 0.52	2.42 ± 0.73	0.91 ± 0.26	1.05 ± 0.22
mean	54 ± 17	2.13 ± 0.73	2.45 ± 0.73	0.91 ± 0.27	1.01 ± 0.21

Renal function measurements were performed 8 weeks after injection of adriamycin, that is after stabilization of proteinuria.

Clearance protocols

After seven days recovery from surgery the clearance experiments were performed. The rats were unrestrained and awake in a metabolic cage. To ensure a stable diuresis during the experiments fifteen hours before the start of the experiment a continuous infusion of a 5% dextrose solution (2 ml/hr) was started via the jugular vein. At 8:00 a.m. a loading dose of 0.08 MBq ^{125}I-iothalamate and 0.16 MBq ^{131}I-hippuran in 300 μl dextrose water was given, followed by a continuous infusion of 0.02 MBq ^{125}I-iothalamate and 0.04 MBq ^{131}I-hippuran per ml dextrose water (2 ml/hr) via the carotid catheter by a constant infusion pump (Harvard Instrument Company, South Natic, USA). After an equilibration period of at least 120 minutes to establish steady state hippuran and iothalamate plasma levels, collection of spontaneously voided urine portions started. Urine was collected by a collection system which separates urine from feces. Following every spontaneous micturition a blood sample of 300 μl was drawn via the jugular catheter. Rats were followed until four clearance periods were completed. Blood was replaced with an equal volume of dextrose water. In the second group of rats ($N = 8$) the bolus and the continuous infusion of the tracers were administered by the intraperitoneal catheter.

Samples. Urine samples were collected in preweighed tubes and volumes were measured gravimetrically. Blood samples were collected in heparinized tubes. The blood samples were immediately centrifuged and a plasma sample of 150 μl was taken. The activities of ^{125}I-iothalamate and ^{131}I-hippuran were determined in 150 μl urine, in 150 μl plasma and in 20 μl standard infusion solution using a two-channel scintillation counter (LKBG Compugamma scintillation counter; Wallace, Finland).

Renal function measurement

Standard GFR was calculated as the urinary clearance ^{125}I-iothalamate, according to the formula:

$$\text{Standard GFR} = \frac{U_{iot} * V}{P_{iot}}$$

where U_{iot} is counts/min per ml urine, V is volume of urine in ml/min, and P_{iot} is counts/min per ml plasma. To correct the standard GFR for inaccurate urine collection, the method as described by Donker et al [13] and Apperloo et al [14] was applied. This correction method relies on simultaneous infusion of ^{131}I-hippuran and ^{125}I-iothalamate, and is based on the following principles. Under conditions of steady state, that is, when infusion rate equals excretion rate with consequently a stable plasma level, the plasma clearance (I*V/P) and the renal clearance (U*V/P) of ^{131}I-hippuran have been proven to be interchangeable in case of perfect urine collection ($r = 0.998$) in humans [13]. Thus, simultaneous infusion of the radioisotopes allows to correct the renal clearance of ^{125}I-iothalamate for incorrect urine collection by using the following formula:

$$\text{Corrected GFR} = \frac{(I_{hip} * V_I/P_{hip})}{(U_{hip} * V_U/P_{hip}} * \text{Standard GFR}$$

where I_{hip} is counts/min per ml of the infusion solution, V_I is volume of the infusion in ml, P_{hip} is counts/min per ml plasma and U_{hip} is counts/min per ml urine, V_U is volume of the urine in ml. Simultaneous infusion of hippuran also gives the opportunity to assess the ERPF, since hippuran is a marker of effective renal plasma flow (ERPF).

Data analysis. The clearance of ^{125}I-iothalamate during a collection period was calculated from the timed urine collection and the bracketed plasma samples. The average of four clearance periods was used to calculate mean GFR. The intra-assay coefficient of variation was measured using the four clearance periods for the individual rats ($N = 17$). Interassay coefficient of variation was measured by comparing measurements on two ($N = 12$) or three ($N = 8$) separate days. A recovery period of at least seven days was allowed between the study days. In the rats ($N = 8$) with renal function impairment three clearance periods were used to calculate mean GFR and intra-assay variation.

All data are expressed as mean ± SD. Statistical analysis was performed by using the matched-pair Student's *t*-test. This was used to compare the differences between the mean standard and corrected GFR for each rat, respectively.

RESULTS

The presence of steady state is a prerequisite for the valid application of this correction method for GFR. The presence of steady state, that is, renal excretion of ^{131}I-hippuran equals infusion rate, can be established by first, stable plasma levels, and second, a complete urinary recovery of the infused ^{131}I-hippuran. Plasma levels were stable; the average consecutive hippuran counts were 383 ± 58, 380 ± 85, 383 ± 109, 399 ± 96 and 390 ± 110 counts/min, respectively. The urinary recovery of ^{131}I-hippuran in the experiments with the intra-arterial tracer infusion, was 90 ± 11%. To test whether this incomplete recovery was due to extrarenal loss of hippuran or incomplete urine collection, we tested our urine collection system. Therefore 20 urine portions ranging from 0.5 to 2 ml were used. We found an average loss of 10 ± 8% urine, due to adhesion to the collection system.

To investigate whether the intra-arterial infusion route could be replaced by an easier intraperitoneal route we tested the latter technique in a second group of animals. The intraperitoneal route, however, resulted only in a 68 ± 8% recovery of hippuran, thus precluding application of the correction method.

Collection time was by design determined by the interval between spontaneous voiding. The voiding interval ranged from 20 to 120 minutes with an average period of 54 ± 17 min at the infusion rate of 2 ml/hr. Urine volume ranged from 0.70 to 3.70 ml

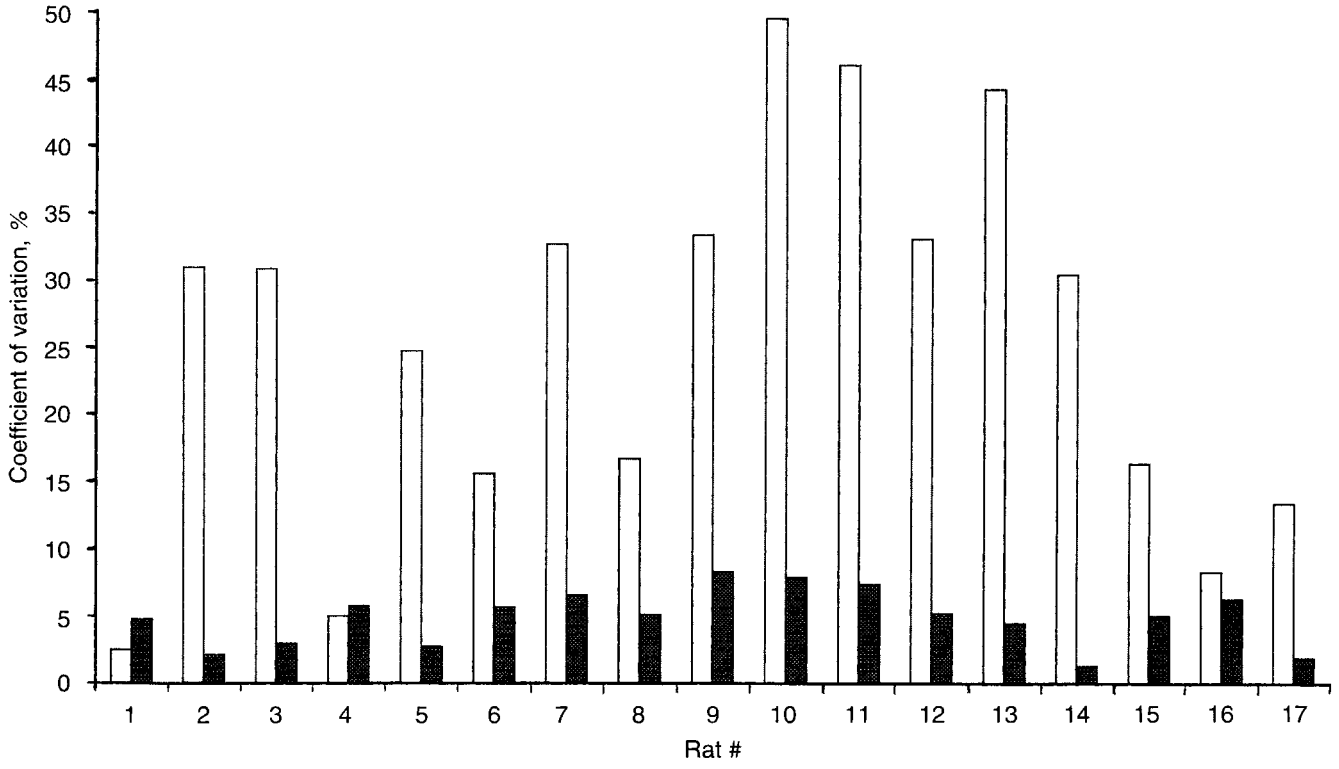

Fig. 1. Coefficient of variation of standard (□) and corrected (■) GFR for each rat.

per voiding with an average of 2.13 ± 0.73 ml. The time course of mean values for duration between voidings, urine volume, urine flow, standard and corrected GFR are given in Table 1. These data show that during the experiment a certain variability between the subsequent collection periods occurs for voiding interval, urine volume, and standard GFR. After application of the correction method the variability in GFR between collection periods tends to decrease somewhat.

Differences between the standard method and corrected method become readily apparent when the individual coefficient of variation of GFR between clearance periods are considered. Figure 1 shows, first, the large intra-individual coefficient of variation (CV) of the standard GFR and, second, a considerable decrease of the individual CV after application of the correction method. Moreover, Figure 1 shows that this reduction in CV of GFR occurs in nearly all rats. This fall in CV was independent of the prevailing GFR. The mean coefficient of variation of GFR fell from 27.4 ± 14.3% to 5.3 ± 2.3% ($P < 0.05$), after application of the correction method. To assess the inter-assay variation, renal function was measured on two or three separate days in a group of 20 rats. In comparison with the standard method, a lower inter-assay coefficient of variation was obtained with the correction method as compared to the standard method (11.0 ± 7.2% vs. 23.4 ± 10.3%).

To investigate whether the correction method was also applicable in rats with impaired renal function we tested the technique in 8 rats with established adriamycin nephrosis. The mean proteinuria in this group was 603 ± 148 mg/24 hr. In these rats, steady state was also established within a time frame of two hours, as indicated by stable plasma levels of ^{131}I-hippuran. Average consecutive counts/min were 565 ± 70, 568 ± 57, 609 ± 75, 589 ± 115, respectively. The second prerequisite for steady state the urinary recovery of ^{131}I-hippuran was also met, as shown by a urinary recovery of 85 ± 27%. The average of three clearance periods resulted in mean standard GFR of 0.54 ± 0.13 (ml/min 100 g body wt) and a mean corrected GFR of 0.59 ± 0.10 (ml/min 100 g body wt). A reduction in variability between the standard and corrected GFR for each individual was also apparent in this group; the intra-assay variation was lowered from 27.9 ± 20.7 to 2.7 ± 1.6% ($P < 0.05$) by application of the correction method.

DISCUSSION

The current study shows, first, that the results of GFR measurement by renal clearance of continuously infused ^{125}I-iothalamate are subject to a large intra-assay variation. Second, we show that correcting GFR by the ratio of plasma and renal clearance of coinfused hippuran considerably reduces the variability. Lastly, we show that intraperitoneal infusion of the tracer results in an incomplete recovery, and, consequently is not applicable for renal function measurement according to this design.

Several clearance techniques have been described to measure renal function in conscious rats. Most methods require correctly timed urine collection. As incomplete emptying of the bladder upon spontaneous voiding can introduce substantial errors in the assessment of urinary clearance of tracer substances, bladder catheters are applied frequently. These, however, have disadvantages such as the need for surgery, and sometimes the requirement of a restrainer [5]. This induces additional stress and thus can affect kidney function. Bladder catheters can also lead to

complications such as urinary tract infections and stone formation [11, 12]. Furthermore, there is evidence that even with the use of a bladder catheter urine collection may be incomplete as a consequence of dead space [17]. To avoid the need of urine collection, the single injection technique was recommended to measure renal function in conscious rats [9]. Single injection techniques assume that the marker is evenly distributed over the extracellular volume and that equilibrium between the sampled plasma and other body fluids is maintained as the kidney clears the marker from the plasma. However, Schachter, Freinkel and Schwartz [18] found that the concentration of inulin in the extracellular fluid only is equal to that in plasma at one point in time after injection. Alternative kinetic models have been advised to overcome these problems of nonequilibrium among fluid compartments. Most of these models still overestimate or underestimate true clearance [19, 20]. Moreover, single injection techniques are not suited for acute pharmacological intervention studies.

For these reasons, we prefer a technique based on the renal clearance of continuously infused ^{125}I-iothalamate. This technique has been used for years in humans, with application of a correction method for incomplete urine collection [13, 14]. This correction method allows to avoid bladder catheterization, but its application is only justified during steady state conditions. In our rat studies we obtained stable plasma tracer levels, and a urinary recovery of ^{131}I-hippuran of 90% and 85%. The major part of the loss of ^{131}I-hippuran, however, appeared to be accounted for by adhesion to the collection system, rather than to extrarenal clearance. Thus, application of the correction method appears to be justified in renal function measurements in rats with normal and moderately impaired renal function.

We did not test the method in rats with severe renal function impairment. When renal function is severely impaired hippuran clearance will be reduced accordingly. Under those circumstances, equilibrium may not be reached within the time frame used here. If so, the lack of equilibrium precludes application of our correction method. To adapt the method for use in rats with severe renal function impairment either the time frame can be prolonged to achieve equilibrium, or alternatively the bolus injection or the infusion rate can be adapted. Such an adaptation, however, would require separate validation.

The intraperitoneal infusion route was not acceptable since only a 68% recovery was achieved. This poor recovery was probably caused by incomplete intraperitoneal absorption of hippuran or by substantial extrarenal clearance as a consequence of increased excretion via the intestinal tract. In conclusion, we were able to perform accurate renal function measurements in spontaneously voiding conscious rats by using a method extensively validated in humans. The lower variation of the corrected GFR compared to the standard GFR, suggests that incomplete bladder emptying, which is common in male rats, accounts for a large part of the intra- and interassay variation in the GFR. The obtained reduction in variability of GFR has two important advantages. First, this method appears to be a suitable technique to assess GFR during acute pharmacological intervention studies. Second, the achieved reduction in variability of the GFR reduces the number of animals needed to study effects of interventions on renal function.

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