Original Articles

Transcutaneous measurement of glomerular filtration rate using FITC-sinistrin in rats

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Abstract

**Background.** Inulin/sinistrin (I/S) clearance is a gold standard for an accurate assessment of glomerular filtration rate (GFR). Here we describe and validate an approach for a transcutaneous determination of GFR by using fluorescein-isothiocyanate-labelled sinistrin (FITC-S) in rats.

**Methods.** Using a small animal imager, fluorescence is measured over the depilated ear of a rat after the injection of FITC-S. The decay curve of fluorescence is used for the calculation of half-life and GFR. The thus obtained transcutaneous data were validated by simultaneously performed enzymatic and fluorometric measurements in plasma of both FITC-S and sinistrin.

**Results.** The results of enzymatic sinistrin determination versus transcutaneous half-life of FITC-S or plasma fluorescence correlated well with each other \((R^2 > 0.90)\). Furthermore, Bland–Altman analyses proved a good degree of agreement of the three methods used. The measurements performed in healthy animals as well as different models of renal failure demonstrate its appropriateness in a wide range of renal function.

**Conclusions.** The transcutaneous method described offers a precise assessment of GFR in small animals. As neither blood and/or urine sampling nor time-consuming lab work is required, GFR can be determined immediately after the clearance procedure is finished. This method, therefore, simplifies and fastens GFR determinations in small lab animals compared to conventional bolus clearance techniques based on blood sampling. A low-cost device for the measurement of transcutaneous fluorescence intensity over time is under construction.

**Keywords:** FITC-S; GFR; transcutaneous measurement

Introduction

Measuring true glomerular filtration rate (GFR) is the most wanted approach for the assessment of renal function. Using inulin or sinistrin (I or S) as an exogenous marker is considered as one of the gold standards for assessing kidney function. A number of alternative methods were introduced, for example, applying radio-labelled markers. The common methods for assessing GFR in small laboratory animals, as required for example in kidney disease models, nephrotoxicity studies or the characterization of genetically modified animals, are cumbersome, invasive and time consuming, due to required multiple blood and/or urine sampling and following laboratory analysis of the samples. A promising approach to simplify GFR determination seems to be the transcutaneous measurement of GFR using exogenous markers \([1–3]\), although none of these approaches is in common use.

With respect to convenience in handling, fluorescein-isothiocyanate-labelled inulin (FITC-I) was established as a GFR marker in rats \([4,5]\). To overcome the poor water solubility of FITC-I, fluorescein-isothiocyanate-labelled sinistrin (FITC-S) was introduced \([6–8]\). Now we describe a method for the transcutaneous measurement of GFR in rats using FITC-S. Our method is based on the measurement of the fluorescence after injection of FITC-S in the interstitial fluid. A small animal imager was used to measure fluorescence intensity over a rat ear. A high correlation between simultaneously performed GFR analysis—transcutaneously and in plasma samples—is shown over a wide range of kidney function.

In the long run, a minimal invasive transcutaneous method could also be a powerful tool to ease GFR determination in humans.
**Subjects and methods**

**Animals**

Male SPF Sprague Dawley (SD) rats about 12 weeks old were purchased from Janvier, France. PCK [9], PKD/Mhm [10] and PKD2mut [11] rats were obtained from our own breeding facility. The animals had free access to standard food and water. All animal experiments were performed according to international and local regulations/guidelines equivalent to the NIH Guide for the Care and Use of Laboratory Animals.

**Marker application and sampling**

For marker application and blood sampling, catheters were inserted into the femoral vein and artery under intramuscular/intraperitoneal ketamine (Ketamin 10%, Essex Tierarznei Corporation, Munich, Germany; 100 mg/kg b.w.) and Xylazine (Rompun 2%, Bayer Corporation, Leverkusen, Germany; 5 mg/kg b.w.) anaesthesia and exteriorized at the back of the neck. Unilateral and 5/6 nephrectomy was performed as published [12]. FITC-S was dissolved in phosphate-buffered saline for injection. Details concerning animal groups and substance dosages are given in Table 2. Blood samples of 0.5–0.7 mL were taken from the arterial catheter. To avoid undue blood loss, the first half of the sample was re-injected after each sampling.

**Technical aspects of the transcutaneous FITC-S determination**

Transcutaneous measurement was performed under anaesthesia. Body temperature was continuously controlled and maintained using a heating mat. To overcome autofluorescence of rat hairs, a depilatory cream was used to remove all hair from the ear. The animal was placed into the CRI Maestro imaging system (CRI Corporation, Woburn, MA, USA) and covered with a non-fluorescent mat in a way that only the ear was in the focus of the CCD camera (Figure 1). Images were taken using a long pass filter starting at 515 nm (Filter set ‘blue’, CRI Corporation) at an exposure time of 10 ms. Checking for background fluorescence, an image was taken before substance injection. After FITC-S application, pictures were taken in intervals of 2 min up to 15 min followed by 5-min intervals up to 120 min. Quantification of the fluorescence signals was performed using the measurement modus of the Maestro 2p20 software (CRI Corporation). Additionally, blood samples for enzymatic and fluorometric assays were taken.

**Fluorometric and enzymatic assays**

Plasma was analysed freshly prepared or after storage at −20°C under protection against light. Fluorescence measured in plasma using multi-well plates (excitation-emission wavelength 485–520 nm; Fluoroskan Ascent, Thermo Labsystems Corporation, Helsinki, Finland) was quantified with the aid of standard curves in pooled plasma. The sugar moiety was determined enzymatically on an EPOS Analyser 5060 (Eppendorf Corporation, Wesseling-Berzdorf, Germany) [7].

**Calculations**

Half-life and GFR were calculated according to the one compartment model using plasma concentration data [13]. For half-life, a single exponential regression was fitted through the data points of the concentration time curve between 45 min (approximately begin of single exponential decay) and 120 min after marker application.

**Determination of the FITC-S conversion factor**

Conversion of half-life data into GFR is needed because the transcutaneous method results in relative emission signals, which do not allow conversion into concentration using e.g. standard curves.

Using compounds excreted by glomerular filtration exclusively, a linear relationship is to be expected between GFR and $t_1/2$. Therefore, a factor can be deduced to convert one parameter into the other.

Sinistrin clearance measured enzymatically was performed separately in 20 healthy awake male SD rats (Table 1). Determination of half-life and GFR was performed according to the one compartment model [13]. Multiplying mean half-life and mean GFR results in the factor of 31.26 (mL/100 g b.w.) for the calculation of GFR normalized on 100 g b.w. and the following formula for conversion:

$$GFR \text{ (mL/min/100 g b.w.)} = \frac{31.26 \text{ (mL/100 g b.w.)}}{t_{1/2} \text{ (FITC-S)(min)}}.$$

For calculation of total GFR (not factored for b.w.), the factor of 31.26 (mL/100 g b.w.) can be replaced by 124.45 (mL).

The formula was applied for GFR determination on transcutaneous, enzymatic and fluorometric $t_{1/2}$ in this study because of the parallel decrease in FITC-S excretion phase independent of the measurement method used. Besides convenience, dosage failures have no influence on GFR values converted from half-life.

**Table 1. Glomerular filtration rate (GFR, mL/min/100 g b.w.) and excretion half-life ($t_{1/2}$, min) measured enzymatically in plasma of awake male Sprague Dawley (SD) rats after intravenous application of FITC-S (mean ± SD)**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>GFR (mL/min/100 g b.w.)</th>
<th>$t_{1/2}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SD healthy awake</td>
<td>20</td>
<td>1.41 ± 0.14</td>
<td>22.12 ± 1.98</td>
</tr>
</tbody>
</table>
Transcutaneous measurement of GFR

Table 2. Glomerular filtration rate (GFR, mL/min/100 g b.w.) and excretion half-life (t₁/₂, min) measured transcutaneously (ear) as well as enzymatically and fluorometrically in plasma of anaesthetized rats after intravenous application of FITC-S

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Dose (mg/kg b.w.)</th>
<th>Enzymatic</th>
<th>Transcutaneous</th>
<th>Fluorometric</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GFR (mL/min/100 g b.w.)</td>
<td>t₁/₂ (min)</td>
<td>GFR (mL/min/100 g b.w.)</td>
</tr>
<tr>
<td>Healthy</td>
<td>7</td>
<td>673</td>
<td>0.90 ± 0.16</td>
<td>35.6 ± 6.9</td>
<td>1.02 ± 0.20</td>
</tr>
<tr>
<td>UNX</td>
<td>6</td>
<td>250</td>
<td>0.68 ± 0.11</td>
<td>47.2 ± 8.1</td>
<td>0.75 ± 0.09</td>
</tr>
<tr>
<td>PCK UNX</td>
<td>12</td>
<td>250</td>
<td>0.56 ± 0.16</td>
<td>59.7 ± 14.1</td>
<td>0.51 ± 0.14</td>
</tr>
<tr>
<td>PKD/Mhm UNX</td>
<td>11</td>
<td>250</td>
<td>0.39 ± 0.09</td>
<td>84.0 ± 20.0</td>
<td>0.37 ± 0.10</td>
</tr>
<tr>
<td>PKD2mut UNX</td>
<td>4</td>
<td>250</td>
<td>0.53 ± 0.10</td>
<td>60.0 ± 10.5</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>5/6NX</td>
<td>5</td>
<td>125</td>
<td>0.18 ± 0.07</td>
<td>203.1 ± 88.6</td>
<td>0.17 ± 0.08</td>
</tr>
</tbody>
</table>

Mean ± SD.

b.w.: body weight; FITC-S: fluorescein-isothiocyanate labelled sinistrin; 5/6NX: 5/6 nephrectomy; UNX: unilateral nephrectomy.

Rats with different hereditary cystic kidney diseases: PCK [9], PKD/Mhm [10] and PKD2mut [11].

In order to economize FITC-S usage, dosage was adjusted regarding to the slower FITC-S excretion of the animal models exhibiting reduced kidney function.

GFR was calculated using the deduced conversion factor of 31.26 (mL/100 g b.w.) (for details see the Subjects and methods section). The animal models were chosen to cover a wide range of renal function, not to present mean values for the respective model. Comparability of GFR as well as t₁/₂ between all three measurement methods is given in a broad range of kidney function and independent of the nature of renal impairment. The findings clearly indicate usefulness of transcutaneous GFR measurement to assess different stages of kidney function.

Results

Transcutaneous measurements of GFR correlate with plasma clearances

For the transcutaneous measurement of GFR, rats were put into a small animal imager, a baseline image of the depilated ear was taken (Figure 1a) and FITC-S was injected. Fluorescence images taken from a rat before injection did not show any signs of fluorescence, when the described settings were used. After injection, the fluorescence intensity increased until ~10 min followed by a decline over time (Figure 1).

The excretion kinetics measured transcutaneously as well as enzymatically and fluorometrically in plasma of anaesthetized healthy rats exhibited a similar decrease (Figure 2). Comparability of excretion half-life and GFR in various groups of rats with normal and reduced kidney function confirmed these findings (Table 2).

Plotting the calculated half-life values (Figure 3a and d) and the derived GFR-values (Figure 3b and e; for the calculation of the GFR from half-life see the Subjects and Methods section) revealed strong linear relationships between the three methods used. Bland–Altman analyses (Figure 3c and f) demonstrated small mean differences (biases) (enzymatic GFR versus transcutaneous GFR = 0.008 mL/min/100 g b.w.; enzymatic GFR versus fluorometric GFR 0.054 mL/min/100 g b.w.) and acceptable 95% limits of agreement [14,15].

Discussion

In order to develop an easy to handle, accurate and precise method for the assessment of GFR, a transcutaneous approach for evaluation of kidney function using FITC-S was established and validated by simultaneously performed plasma-clearances in rats by analysing the excretion of FITC-S over time in a small animal imager.

Transcutaneous fluorescence measurement is advantageous because no blood and urine sampling or time-consuming laboratory work is required and results are obtained immediately after the clearance procedure is finished.

Our clearance experiments in rats cover a wide data range and demonstrate the equivalence of the transcutaneously measured GFR with the simultaneously performed plasma clearance determined enzymatically and fluorometrically. Furthermore, the transcutaneous measurement of half-life and its transformation into GFR using a conversion factor are a suitable method to assess GFR in a wide range of kidney function. This notion is supported by the comparability of GFR values with reported data [1,16,17].
The impact of anaesthesia on the measured GFR values is remarkable. The healthy awake animal group showed a mean enzymatic GFR of $1.41 \pm 0.14$ mL/min/100 g b.w. (Table 1) compared to $0.90 \pm 0.16$ mL/min/100 g b.w. in the healthy anaesthetized rats (Table 2). As the aim of the study was the validation of the transcutaneous technique against the enzymatic standard procedure and not the GFR determination itself, no effort was taken to overcome this influence. In contrast, no changes in the general characteristics of the elimination kinetics in plasma could be observed in all examined animal models showing reduced kidney function as well as under anaesthesia. The change of the fast initial decrease in concentration into a single exponential slower decrease in concentration could be observed after $\sim 45$ min in all models, indicating that the substance distribution into the interstitial fluid is basically driven by diffusion processes and not the circulatory flow.

In our study, we could not detect fluorescence in blood vessels of the ear (Figure 1; dark lines on the ear). This can be explained by the high absorption of haemoglobin at the emission maximum of FITC at 520 nm. Both relatively long- and time-dependent rise up to the maximum fluorescence intensity in the rat ear and the absence of fluorescence in blood vessels strongly suggest interstitial fluid as distribution compartment, where FITC-S is measured transcutaneously. The free diffusion of fructosanes into the interstitial fluid was described previously [18]. This notion is supported by the comparability of the elimination kinetics of FITC-labelled poly-D-lysine measured transcutaneously in one rat [19]. The parallel decrease in transcutaneous fluorescence signal and marker concentration in plasma indicates a fast equilibration of the marker in blood and interstitial fluid.

The one compartment model for GFR determination was chosen despite the known drawbacks that total equilibrium of the substance between blood and ISF after a certain time has to be assumed, and the fact, that the volume of distribution cannot be assessed reasonably as the initial fast decrease in concentration in plasma is not considered, which can also lead to an overestimation of GFR. It is, however,
the only model, which can be used, if GFR assessment is performed transcutaneously in the interstitial fluid and not in plasma samples, as the transcutaneous data do not allow drawing any conclusion about the initial fast concentration decrease in plasma, required for the use of the two compartment models. The degree of potential overestimation of GFR, by the method used, needs to be determined by validation against a renal clearance technique, based on blood and urine sampling in further studies. An advantage to evaluate kidney function on the basis of an excretion half-life (which is only possible using a one compartment model) is its independence from dosage errors that are especially crucial if small marker volumes are injected via long catheters into small animals.

I/S and fluorescein are used in clinical practice for decades with very rare reports about unwanted effects [6,20,21]. Due to the excellent safety profile of both substances, the high water solubility of FITC-I and the demonstrated usefulness as a GFR marker, our method offers an advantage even over recently published transcutaneous methods using either FITC-I [1], europium-DTPA-monoamide complexes [2,3] or the isotopic GFR marker $^{99m}$Tc-DTPA [22].

The major problem with FITC-I is its poor water solubility [6], while europium-DTPA-monoamide is a heavy metal chelate with very limited information about toxicity and a considerable degree of non-renal clearance [2,3]. The problem of isotopic GFR markers is the high costs, the lack of availability and the lack of acceptance of radio-labelled drugs in general. Besides the isotopic method, there are also no detailed data available comparing such clearances with an established GFR marker covering a wide range of renal function simultaneously [2,3].

Yu et al. [1] had been able to detect FITC-I only in vessels through the skin, but only for ~6 min, not taking the diffusion into the interstitial fluid into account. Then the signal disappeared. This discrepancy may be explained by the fairly low dose, the high absorption of haemoglobin at 520 nm and the sensitivity of the measurement system used.

Both in the study of Yu et al. [1] and in ours, expensive hardware is used. Low-cost devices are clearly needed either as a needle-type sensor or a sticky plaster. Pilot tests using a demonstrator of a miniaturised low-cost device were encouraging.

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Conflict of interest statement. D.S., M.S., J.P. and N.G. are inventors in a patent application covering this subject. N.G. is one of the managing directors of InnovationLab GmbH.

References


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