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Laboratory Notes

Fluorescein-labeled sinistrin as marker of glomerular filtration rate

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Abstract

There is an obvious and growing medical need for an accurate and easy to handle determination of glomerular filtration rate (GFR) for a broad spectrum of indications. Newly synthesized fluorescein-isothiocyanate (FITC)-sinistrin (FS) with various degrees of labeling was selected by its physicochemical properties and good tolerability out of a number of dye-labeled compounds intended for use as GFR markers for characterization of its pharmacological profile. With respect to solubility FS is more convenient in handling compared to FITC-inulin (FI). Up to 100 mg ml^{-1} of FS can be dissolved in aqueous solvents at room temperature, whereas FI can only be solubilized after warming up to 55 °C. This reveals a considerable advantage of FS over FI in preparation of galenical formulations for intended i.v. application. A fluorometric method was established to determine FS concentration in blood serum with a comparable accuracy to the established enzymatic method for polyfructosanes. Similar concentration time curves in blood serum of FS measured fluorometrically and enzymatically suggest no relevant change of pharmacokinetic behavior by dye labeling. This notion is supported by the rapid renal and missing of biliary excretion. On the basis of these results, FS is superior in handling to the available GFR markers and makes it highly interesting as a novel diagnostic drug.

Keywords: Glomerular filtration rate; Fluorescein-isothiocyanate-sinistrin; GFR marker; Renal function; Rats

1. Introduction

Annually in about 60 patients out of 1 million population end-stage renal disease is diagnosed due to diabetes, hypertension and kidney disease [1–5]. Life-saving therapeutic intervention are dialysis and kidney transplantation, both cost-intensive and resulting in reduction of quality of life. Additionally, life expectancy is reduced dramatically [6,7]. For therapeutic improvement of this situation an accurate evaluation of the kidney function is a prerequisite [8]. Aggressive pharmacotherapy in a wide range is available and/or dietetic interventions could be initiated in due time [9–14].

Determination of glomerular filtration rate (GFR) using the classical inulin clearance is the most accurate method and therefore the gold standard for assessing kidney function in humans. This method requires high expenditure, is time consuming both for the patients and laboratory personal [15–18]. Using radio-labeled markers, GFR measurement becomes easier to perform, but the acceptance of application of radioactive compounds is low in the population in general [19–21]. Alternatively, clearance of endogenous creatinine is used as a surrogate marker which is known to be imprecise. Furthermore, plasma creatinine is a widely used surrogate parameter which only allows rough estimation of GFR. The major disadvantage of this method is its low sensitivity [8,22–24]. Additionally, the height of serum creatinine depends on total muscle mass and dietary habits.

The disadvantage of these methods results in the fact that the GFR is currently determined only rarely in clinical practice which causes a dramatic delay in early diagnosis of renal impairment [8]. There is a great clinical demand for an accurate method of performing GFR measurement. GFR is considered to be an important component in diagnosis and overall management of therapy in situations summarized in Table 1. "The design and testing of new methods that will consistently identify diabetic patients at the earliest stage of renal damage" is addressed in the consensus statement of the

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Table 1
List of indications and medical fields with importance of GFR for diagnosis and overall management of therapy

Diabetes
Hypertension
Renal diseases
Glomerulonephritis
Glomerulosclerosis
Nephrotic syndrome
Renal artery stenosis
Polycystic kidney disease
Dialysis
Kidney transplantation (donor and recipient)
End-stage liver disease
Intensive care
Pediatric patients with renal disease
Geriatric medicine (dose adjustment)
Monitoring of drug therapy with highly toxic agents primarily excreted renally (e.g. cancer chemotherapy, immunosuppression)
Monitoring of changes in kidney function as a result of therapy (for assessment of effectiveness of drug therapy)

Clinical Practice Recommendations of the American Diabetes Association 1996 [25].

In the following the synthesis of a novel GFR marker, called fluorescein-isothiocyanate (FITC)-sinistrin (FS) and its measurement to determine GFR is described in detail. The measurement is based on the i.v. injection of FS. Its time-dependent decrease in blood can be measured by use of a conventional fluorometer. The determination of GFR using FS fulfils the requirements to assess kidney function accurately in a manner convenient to both patient and physician [8,18,26]. The decrease in effort will enable a greater acceptance of this method.

2. Chemistry

The synthesis of FS is done in a one-step reaction starting from sinistrin and commercially available FITC (Fig. 1). Purification in (multi-g) lab-scale is performed by a combination of precipitation and chromatography. Sinistrin is a natural product and contains ca. 97% of fructose and ca. 3% of glucose (Glu). The degree of labeling of FS amounted to about 0.7–3% depending on the amount of FITC used in synthesis.

3. Characterization of FS as an improved marker of GFR

3.1. Physicochemical and pharmacological properties

For the last 80 years inulin clearance has been the gold standard for the determination of GFR [15–18]. Inulin is a polyfructose molecule of poorly defined molecular weight ranging from 1000 to 20,000 Da with a mean value at around 5000. It is further characterized by poor solubility in water and a need of heating before injection. This poor solubility may be the reason for the sometimes poor tolerance in humans [27]. It is also of note that immunologists use high doses of inulin for the activation of the complement pathway in vitro indicating potential for unwanted effects when used as GFR marker [28]. Immunological reactions have rarely been described for both sinistrin and inulin, but they need further clarification for FS [29–31].

As an alternative sinistrin (Polyfructosan S) was developed. It is characterized by high solubility and a more narrow molecular weight range between 2000 and 6000 with an average around 3500 [32].

The common problem with both inulin and sinistrin, however, is that the biochemical determination is fairly difficult and time consuming [15–18,33]. For this reason other markers like radio-labeled inulin or EDTA are used [19–21].

In order to find an alternative marker for the standard inulin clearance we synthesized dye-labeled compounds intended as GFR markers [34]. Labeled sinistrin with FITC resulting in FS (for details see Section 5.1) was selected out of a number of newly synthesized markers because of its good tolerability and ease of handling. The high solubility of FS in water

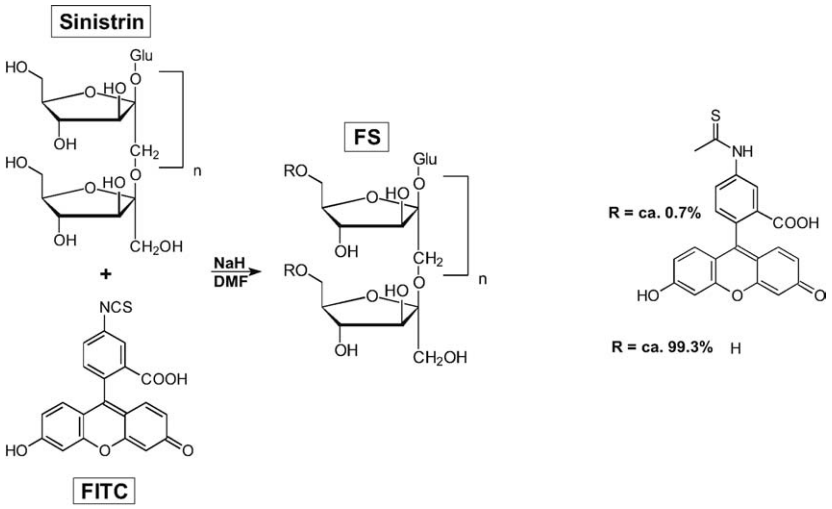

Fig. 1. Scheme of FS synthesis.

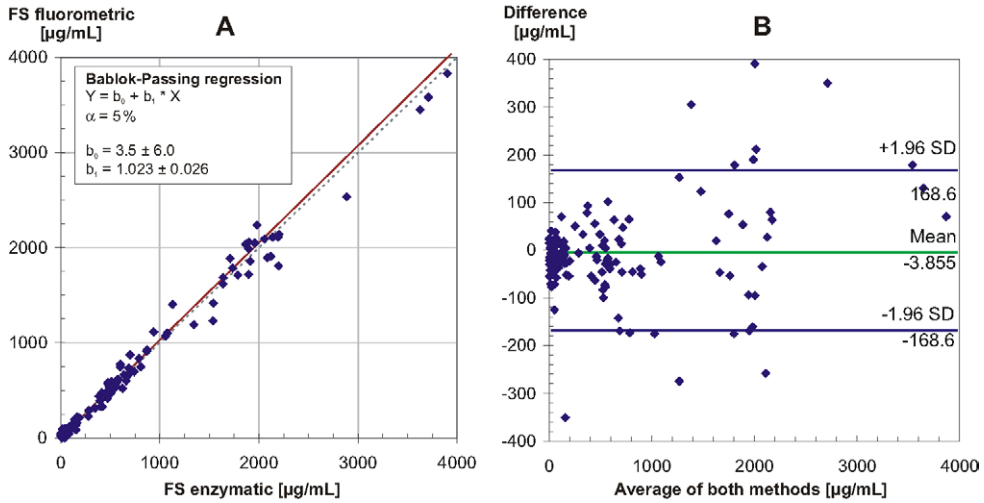

Fig. 2. Method comparison of FS measured fluorometrically and conventionally using standard enzymatic method. Comparability of fluorometric method with "gold standard" is shown using Bablok–Passing regression (A) and Bland–Altman analysis (B).

at room temperature makes the compound a superior candidate for parenteral application compared to FITC-inulin (FI). Whereas FS and sinistrin is soluble in hydrous solvents (saline and phosphate buffered saline) up to 100 mg ml^{-1} at room temperature, FI and inulin require warming up to 55 °C.

FS is characterized as a highly soluble, amorphous lyophilisate. With increasing water content the substance becomes a yellow liquid with decreasing viscosity. Its concentration can be readily measured by standard fluorometers. Additionally the biochemical determination of the polyfructosane moiety [35] is still possible and gives the same results as the fluorometric determination. The comparison of the enzymatic and fluorometric detection of FS concentrations showed a high correlation using linear regression (Fig. 2A) which is supported by Bland–Altman analysis (Fig. 2B). This finding is true in low concentration range predominantly, which demonstrates, that FS can be quantified fluorometrically in an adequate manner compared to the established enzymatic method [35].

The concentration time curves of FS (Fig. 3) in serum of rats after i.v. application are comparable. These indicate no change of excretion behavior of FS by labeling. Furthermore the identical course of the concentration time curves measured enzymatically and fluorometrically demonstrates, that the fluorometric quantification is an adequate method for in vivo use of FS.

When injecting 30 mg of FS into rats, 27.5–29 mg could be recovered in urine within 24 h. This means that more than 90% are excreted renally within 24 h (Table 2). FS concentration in bile fluid exceeded the detection limit very slightly 15 and 30 min after i.v. application only suggesting no relevant biliary excretion of FS (data not given).

These findings of in vivo experiments strongly indicate, that the labeling did not affect the biological "quality" of the

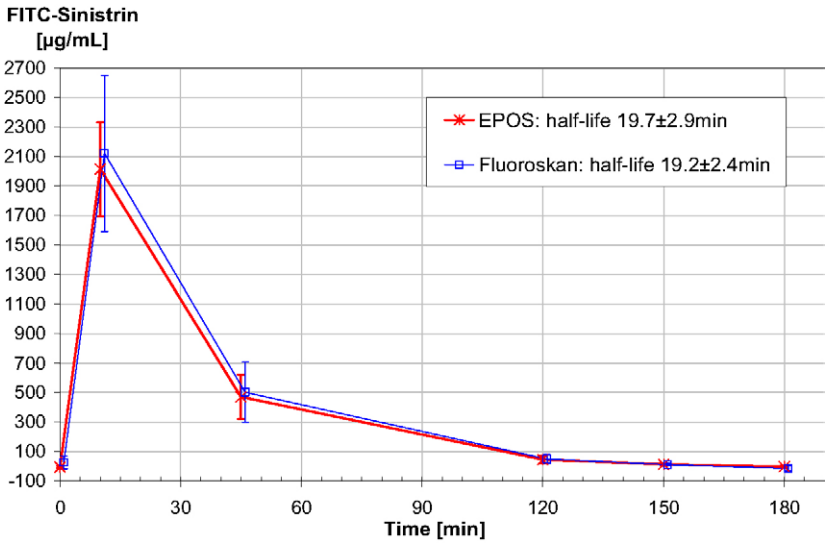

Fig. 3. Concentration time curve of FS after i.v. injection of 750 mg kg^{-1} dissolved in phosphate buffered saline (300 mg ml^{-1}) in male Sprague–Dawley rats ($n = 7$, mean ± S.D.).

Table 2
Renal excretion of FS in male Sprague–Dawley rats after i.v. application of 30 mg per animal measured fluorometrically and enzymatically. Cumulative excretion is given in percent of dose injected

Urine sampling intervals (h)	Fluorometric detection			Enzymatic detection		
	Animal 1 (%)	Animal 2 (%)	Animal 3 (%)	Animal 1 (%)	Animal 2 (%)	Animal 3 (%)
0–1	61.0	68.8	46.5	61.1	78.7	51.4
1–3	87.0	80.2	79.7	90.9	88.4	89.3
3–6	90.4	85.5	83.3	94.1	92.8	91.4
6–12	92.5	87.3	85.5	96.1	94.3	93.6
12–24	93.5	88.3	86.7	96.7	95.0	94.7

molecule with respect to use as GFR marker. First trials showed that FS can also be measured by using test strips. In principle it is also possible to determine FS transcutaneously, as first studies in rabbits demonstrated.

3.2. Tolerability

Pilot toxicity studies were performed with repeated i.v. injections into rats. The animals did not show any sign of discomfort during or after injection of FS. In liver, kidneys, spleen, pancreas and lung no drug-induced damage could be observed histomorphologically. Additionally we could not find any deposits of fluorescence-labeled material in relevant organs, i.e. lung, spleen, liver and kidney. This contrasts with our findings in rats after FI (Sigma Aldrich) injection, described as marker of kidney function in mice and rats [36,37] where considerable deposits of FITC staining material were found in lung, spleen and liver. Furthermore the animals often showed signs of discomfort shortly after injection.

4. Conclusion

Disturbed kidney function resulting in end-stage renal disease is a frequent complication in patients suffering from diabetes, hypertension, glomerulonephritis and other medical fields as listed in Table 1 [1–3]. An early detection of impaired kidney function is a pre-condition for successful therapeutic intervention [8]. GFR reflects kidney function and is, therefore, appropriate for diagnosis and for monitoring therapeutic efficacy [15–17]. The GFR markers available are low in acceptance and convenience to both patients and physicians because of e.g. radioactive labeling, troublesome analytical procedures and high expenditure [19–21]. There is an obvious and growing medical need for an accurate determination of GFR. The procedure for such GFR determination has to be accurate, easy in handling and readily available [8]. The novel compound FS meets these requirements by its physicochemical properties, pharmacological profile as well as good tolerability. The fluorometric method established for determination of FS concentration in blood serum has a comparable accuracy to the established enzymatic method for polyfructosanes [35]. The comparable course of the concentration time curves in blood serum of FS following i.v. application in rats measured fluorometrically and enzymatically does not give a hint for relevant changes of the pharmacokinetic behavior by labeling. This notion is supported by high urinary excretion rate, but no biliary excretion. FS was well tolerated after single and multiple application. Histomorphological investigations of various organs did not give any hint for drug deposition or drug-induced intolerabilities. All in all, FS is superior in handling to the available GFR markers, even to those based on radioactive label [15–23]. High medical need and superiority to standard methods make FS highly interesting as a novel diagnostic drug. This outstanding superiority lets expect a high acceptance rate both by physicians and patients.

5. Experimental protocols

5.1. Synthesis

FITC (~1.5% loading based on available sugar monomers).

Seven grams sinistrin are added to 280 ml DMF at 35 °C and stirred under N_2 until all material is dissolved. After cooling to RT, 2.6 g NaH (60% susp.) is added in small portions, upon which the solution becomes slightly yellow and more viscous. The solution is warmed to 45 °C and stirred for 30 min, and another 30 min at 55 °C. Under rapid stirring, a solution of 0.25 g FITC in 70 ml DMF is added all at once. The solution is stirred at 55 °C for 1 h and overnight at RT. It is then cooled to 5 °C and a solution of 3.34 g NH_4Cl in 15 ml H_2O is slowly added under rapid stirring. The solvent is removed under vacuo in a rotary evaporator (bath temperature 40–45 °C), leaving ~16 g of a solid residue. This is dissolved in 60 ml ethanol/water (1:1) and precipitated upon slow addition of 250 ml acetone. An orange/red oil remains on the flask's surface from which the solvents can be decanted, leaving 14.4 g of crude product. For further purification, 13.4 g of this is dissolved in 500 ml ethanol/water (1:1) and 300 ml silica (Merck, silica 60) is added, and the solvent removed under vacuo. This material is added on a column packed with silica/ethanol. On eluation with 1.9 l of ethanol, some clearly visible organic impurities eluate. The main fraction eluates using 2.1 l ethanol/water (1:1), yielding 6.1 g (~86%) as a highly viscous oil. To transfer this into a more handable form, the material is dissolved in 400 ml H_2O at 35 °C, filtered, divided into two 500 ml one-necked flasks and freeze-dried

to yield orange flakes. The IR and UV spectra distinct the material from its starting material:

IR: ν [cm^{-1}] = 3282, 2932, 1591, 1327, 1121, 1016, 929, 816; UV (1.2 g l^{-1} H_2O): E_{491} = 0.408.

By essentially the same procedure, using 0.5 g of FITC, a material with a higher molar loading of the dye (about 3%, based on available sugar monomers) is obtained.

IR: ν [cm^{-1}] = 3279, 2934, 1591, 1328, 1112, 1015, 929, 816; UV (1.2 g l^{-1} H_2O) E_{490} = 0.717.

5.2. Fluorometric determination

To measure concentration of labeled FS fluorometrically blood serum was transferred to multi-well plates. Fluorescence was determined at different pairs of excitation–emission wavelengths (530–620, 485–520, 390–520, 355–520, 355–460 nm) using a Fluoroskan Ascent (Therma Labsystems). The pairs of wavelengths where chosen to compensate disturbing effects from protein and to approximate the nonlinear fluorescence response with a higher accuracy using a multivariate calibration model. For further evaluations and a simplification of the calibration procedure standard calibration curves were measured using serum and respective dilutions with phosphate buffered saline.

5.3. Enzymatic determination

The polyfructosane moiety of FS was determined in blood serum using a photometric analyzer (EPOS Analyzer 5060, Eppendorf). The procedure was modified from enzymatic inulin determination described by Kuehnle et al. [35]. To oxidize native glucose and hydrolyze sinistrin glucose oxidase (Roche) and β-fructosidase (Novo Nordisk) were added to 100 μl of blood serum, mixed and incubated for 60 min at 37 °C. An aliquot of the reaction mixture was transferred to the autoanalyzer using the GLUCO-QUANT® enzyme kit (Roche Diagnostics/Hitachi) after adding phosphoglucose isomerase to reagent solution 1.

5.4. Pharmacological methods

5.4.1. Animals and treatment

Male SPF Sprague–Dawley rats about 12 weeks old (breeder: Janvier, France) were obtained for the study. Feed (Ssniff R/M-H diet, Ssniff Versuchstierdiäten GmbH, Soest, FRG) and water (from drinking bottles) were available ad libitum. Animals' health was monitored daily.

For drug application and blood sampling catheters were inserted into the femoral vein and artery under intramuscular/intraperitoneal Ketamin (Hostaket®, Parke-Davis, Germany; 100 mg kg^{-1} body weight) and Xylazin (Rompun® 2%, Bayer, Germany; 5 mg kg^{-1} body weight) anesthesia and exteriorized at the back of the neck. The animals were allowed to recover for 2 days. The drugs dissolved in phosphate buffered saline were injected slowly.

5.4.2. Sampling of blood, bile fluid and urine

Blood samples of 0.5–0.7 ml were taken from the arterial catheter. Avoiding too much blood loss saline together with about 0.3 ml blood were reinjected after each sampling. Serum was obtained by centrifugation for direct analytical measurement or storage at –20 °C under continuous protection against light.

To collect bile fluid a catheter (B.D. Microlance, outer diameter: 0.6 mm) was inserted into the bile duct under anesthesia described above. Sample portions were cut in an interval of 30 min with an additional sampling after 15 min over a time period of 180 min. Urine was collected after i.v. application of FS into conscious rats using metabolic cages. Measuring procedure were the same described for blood serum.

5.4.3. Histomorphological investigations

Part of the organs were fixed in 3% buffered formaldehyde and further processed for paraffin embedding and hematoxylin–eosin (H&E) staining and part of the organs were snap frozen and processed for the detection of deposits of FITC-sinistrin (FS) or FI using a fluorescence microscope.

5.5. Computational procedures

In order to evaluate the Fluoroskan measured data a multivariate calibration model was constructed with the help of partial least squares (PLS) regression. The calculations were done using "SCAN Software for Chemometric Analysis, Version 1.1, Minitab Inc." and "Microsoft Excel 2002, Microsoft Corp.". The calibration model was build with 114 randomly selected samples from in vivo animal experiments. Fluoroskan data and protein concentrations [38,39] were fed into the regression algorithm. As reference values FS concentrations measured enzymatically were used. Because of the interpolation of nonlinear fluorescence behavior by using the linear PLS method a relatively large number of components was chosen. An explained variance of 92% with for of six possible principal components was defined as sufficient. The evaluation of the unknown in vivo samples was done in Excel using the above described calibration model. Bland–Altman analysis and Bablok–Passing regression were used to evaluate the comparability of both detection methods [40,41].

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