Glomerular permeability during ACE inhibition
Hemmelder, Marcus Henricus

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A comparison of analytical procedures for measurement of fractional dextran clearances

Hemmelder M.H., de Jong P.E., de Zeeuw D.

with technical assistance of Jilderda J.F., Hamminga J.E.
and van der Wal-Hanewald J.W.J.T.

Introduction

Measurement of fractional clearances of neutral dextrans with broad molecular size distribution is a well-known and appropriate method to obtain insight in the size selective function of the glomerular filtration barrier [1-3]. In contrast to endogenous proteins, clearance of dextrans is not influenced by charge dependent filtration or by reabsorption or secretion by tubules [1-2]. Previous clinical studies which analyzed fractional dextran clearances attributed significantly to the understanding of the pathophysiology of the glomerular filtration barrier [4-45]. In short, nephrotic range proteinuria of various origin is associated with a loss of glomerular size selectivity reflected by an increased fractional clearance of large dextrans and a decreased fractional clearance of small dextrans in comparison to normal conditions. By means of a heteroporous model of glomerular size selectivity, increased fractional clearances of large dextrans can be explained by an increased fraction of the filtrate which passes without restrictions through large pores in the glomerular basement membrane. Loss of ultrafiltration capacity due to reduction of hydraulic permeability of the glomerular capillary wall reduces filtration of small dextrans through the second pathway of small and size-restrictive pores [14].

The laboratory procedure as described by Granath et al. forms the backbone of measurement of fractional dextran clearances [46-47]. In short, polydisperse dextrans in plasma and urine samples are separated in narrow fractions according to their molecular size by gel permeation chromatography (GPC) on precalibrated columns, whereas the dextran concentration in the collected molecular size fractions is estimated by the anthrone assay [48]. This allows an accurate calculation of the renal dextran clearances relative to the glomerular filtration rate over a broad range of molecular sizes. Most studies on renal dextran sieving in human nephropathy used this laboratory procedure, whereas several more recent studies introduced an alternative laboratory approach for the measurement of fractional dextran clearances (Table 1). In retrospect, studies differ with respect to methods of dextran infusion (bolus with or without continuous infusion of different dextrans), sample preparation (no protein precipitation or protein precipitation with different agents in different concentrations), calibration (different number and polydispersity of standard dextrans), size-exclusion chromatographic (GPC or HPLC), and dextran detection (anthrone assay or on-line refractometry).

To date, no systematic comparison has been published on those different laboratory methods, except for one comparison of different methods of dextran infusion [49]. In the present study, we therefore compared different methods of sample protein precipitation, column calibration, size-exclusion chromatography, and dextran detection. We secondly analyzed whether use of different dextran size-exclusion chromatography and dextran detection methods induce differences in the measurement of fractional dextran clearances in healthy subjects and patients with non-diabetic proteinuria.
Table 1. Review on laboratory procedures used in clinical studies which analyzed glomerular size selectivity by fractional dextran clearances.

<table>
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<tr>
<th>Author</th>
<th>Year</th>
<th>Infusion</th>
<th>Prec</th>
<th>Calibration</th>
<th>Chrom</th>
<th>Detect</th>
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<td>GPC</td>
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<tr>
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<tr>
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<td>D9-D14 (Granath)</td>
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<td>GPC Anthron</td>
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<td>Zietse</td>
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<td>TCA 10%</td>
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</table>

Abbreviations are: Prec = precipitation method; ZnSO4 = zinc sulfate; TCA = trichloroacetic acid; us = unspecified precipitation method; Plasma = only plasma precipitation; Calibration = standard dextrans for calibration; Chrom = size-exclusion chromatography method; Detect = dextran detection method; RI = on-line refractometry.
Methods

Sample preparation

Blood and urine samples are deproteinized before assay to prevent interference of proteins during dextran detection and to prevent contamination of polymer HPLC columns. We analyzed protein and dextran recovery after sample protein precipitation by trichloroacetic acid (TCA), at low and high concentration, and zinc sulfate. For this purpose, we used plasma spiked with a 2 mg/ml equivalent mixture of dextran-40 (Rheomacrodex®, Pharmacia, Uppsala, Sweden) and dextran-70 (Macrodex®, Pharmacia, Uppsala, Sweden) and urine from a proteinuric patient with a dextran concentration of 2.5 mg/ml, which can be considered an average concentration. On the one hand, 100% TCA was added to samples to a final concentration of 5 or 20%. After centrifugation during 5 minutes (16000 rpm), supernatants were titrated to pH 7.0 with NaOH. On the other hand, a mixture of 1 ml 10% zinc sulfate, 1 ml 1N NaOH, and 1 ml mobile phase were added to 1 ml sample followed by centrifugation during 5 minutes (16000 rpm). Supernatants had a pH of 7.0.

Size-exclusion chromatography

Gel permeation chromatography (GPC) was performed on a 90 by 1.5 cm Sephacryl S-300 column (Pharmacia, Uppsala, Sweden). A 0.01 M Tris buffer with 0.15 M NaCl and 1 mM EDTA at pH 7.0 was used as mobile phase. A 1.0 ml sample was applied to the column, whereas fractions of approximately 2.9 ml were collected in polystyrene tubes (Greiner, Alphen aan de Rijn, The Netherlands) in a Biorad 2128 fraction collector (Biorad, Hercules, CA, USA). This setup allowed us to perform two assays during 24 hours.

High performance liquid chromatography (HPLC) was performed on two polymer columns in series (BIO-Gel SEC 30XL and BIO-Gel SEC 40XL, each 300 by 7.8 mm; Biorad, Hercules, CA, USA) which were protected by a guard column (BIO-Gel XL, 40 by 6 mm; Biorad, Hercules, CA, USA). The other components which we used were a Jasco 880-PU HPLC pump (Jasco, Tokyo, Japan), a Waters U6K injector (Waters Corporation, Milford, MA, USA), a Biorad 1775 refractive index monitor (Biorad, Hercules, CA, USA), and a Biorad 2128 fraction collector (Biorad, Hercules, CA, USA). A 0.02 M phosphate buffer at pH 7.0 was used as mobile phase (0.5 ml/min). The pressure ranged between 20 and 30 kg/cm². A 0.1 ml sample was injected, whereas fractions of approximately 0.43 ml were collected in polystyrene tubes. This setup allowed us to perform six assays during 24 hours.

Dextran detection

Dextran concentration in each fraction from GPC and HPLC assays was determined using the anthrone assay [48]. Fractions of plasma and urine from one subject were analyzed in the same anthrone assay in which 0.4 ml sample was mixed with 1 ml anthrone reagent. The mixture was immersed in a boiling water bath for 16 minutes. The absorbency at 625 nm was converted to dextran concentration
by the simultaneous measurement of absorbency of dextran samples with known concentration over a range from 0 to 0.11 mg/ml.

During HPLC, dextran concentration in plasma and urine samples was also measured by on-line refractometry [50]. The detection signal was digitized and analyzed using commercially available software (JCL6000, Jones Chromatography Inc, Littleton, Colorado, USA).

Table 2. Molecular weight characteristics of standard dextrans.

<table>
<thead>
<tr>
<th></th>
<th>M_w (kD)</th>
<th>M_n (kD)</th>
<th>M_w/M_n</th>
<th>(M_w*M_n)^½ (kD)</th>
<th>R_st1 (Å)</th>
<th>R_st2 (Å)</th>
<th>R_st3 (Å)</th>
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</thead>
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<tr>
<td>F8</td>
<td>10.2</td>
<td>5.7</td>
<td>1.80</td>
<td>7.6</td>
<td>20.7</td>
<td>23.7</td>
<td>27.6</td>
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<tr>
<td>F40</td>
<td>36.7</td>
<td>24.3</td>
<td>1.51</td>
<td>29.9</td>
<td>39.0</td>
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<td>48.0</td>
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<tr>
<td>F75</td>
<td>71.0</td>
<td>39.4</td>
<td>1.80</td>
<td>52.9</td>
<td>50.8</td>
<td>58.1</td>
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<td>67.1</td>
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</table>

Abbreviations are: M_w = mass average molecular mass; M_n = number average molecular mass; M_w/M_n = polydispersity; (M_w*M_n)^½ = molecular weight correction for polydispersity; R_st1 = Stokes radius calculated from 0.33 * ((M_w*M_n)^½)^0.463 as in the present study; R_st2 = Stokes radius calculated from 0.33 * M_w^0.463 according to Granath et al. [46]; R_st3 = Stokes radius calculated from 0.448 * M_w^0.437 according to Oliver et al. [52].

**Calibration**

GPC and HPLC systems were calibrated at 6 week intervals using three commercially available standard dextrans F8, F40, and F75 (Serva, Heidelberg, Germany) with specified weight average molecular weight (M_w) and number-average molecular weight (M_n) (Table 2). The void volume V_0 of the gel column was estimated at GPC by the elution volume of blue dextran (Pharmacia, Uppsala, Sweden; M_w 2000 kD) and at HPLC by the elution volume of dextran-500 (Pharmacia, Uppsala, Sweden; M_w 500 kD). The total volume of the gel column V_t was estimated at GPC by the elution volume of vitamin B12 (M_w <1 kD) and at HPLC by the elution volume of glucose (M_w < 1 kD). The fractional volume available to the solute (K_AV) was calculated from K_AV = (V_e - V_0) / (V_t - V_0), where V_e is the elution volume of the solute [51]. Effective molecular radii for the individual dextran fractions were estimated from the linear relationship between K_AV and the Stokes radius (R_st)
Laboratory procedure to measure dextran

of the standard dextrans [46]. As in most previous studies which used standard dextrans with high polydispersity (M_w/M_n > 1.3), R_{st} was related to molecular weight by the formula: 

\[ R_{st} = 0.33 \cdot M^{0.463} \]  

[46]. M in this formula represents the molecular weight assigned to the peak position in the elution pattern of a standard dextran. We calculated M of each standard dextran by the formula 

\[ M = (M_w \cdot M_n)^{0.5} \]  

to correct for polydispersity of standard dextrans [50]. In the results section the influence of this correction on fractional dextran clearances will be evaluated. In addition, application of another relation between R_{st} and molecular weight (R_{st} = 0.49 \cdot M^{0.437} [52]) derived from calibration with standard dextrans with very low polydispersity (M_w/M_n < 1.15) will be evaluated with respect to fractional dextran clearances.

In an additional experiment, we compared three different sets of standard dextrans (Table 2) by performing four consecutive calibrations at the HPLC system with the anthrone assay for each set of standard dextrans.

**Variance of dextran assay**

We analyzed the variance of GPC and anthrone assay, HPLC and anthrone assay, and HPLC with on-line refractometry by 10 consecutive assays of a plasma sample spiked with a 2 mg/ml equivalent mixture of Rheomacrodex® and Macrodex®, and an urine sample from a proteinuric patient with a dextran concentration of 2.5 mg/ml. This allows calculation of variance of dextran concentration in plasma and urine, and the urine-to-plasma index of dextran concentration at each molecular size for each used method. Recovery tests showed that dextran recovery during assays of plasma and urine supernatants were not significant different from 100%.

In addition, the variance of the anthrone assay was tested by 20 consecutive measurements of mobile phase samples spiked with dextrans with concentrations from 0.00 to 0.11 mg/ml during one assay.

**Dextran clearance studies**

Dextran clearances were studied in 15 healthy subjects and 17 patients with proteinuria of non-diabetic origin. All healthy subjects denied a history of renal disease, hypertension, or diabetes. At the time of evaluation each healthy subject was found to be normotensive, normoglycemic, and without proteinuria. Patients had biopsy proven non-diabetic renal disease (8 focal segmental glomerulosclerosis, 6 membranous glomerulopathy, 1 IgA nephropathy, 1 minimal change disease, and 1 membranoproliferative glomerulonephritis), diastolic blood pressure below 110 mmHg, creatinine clearance more than 40 ml/min, proteinuria more than 3.0 g/day, and no need for concomitant medication. Patients with diabetes mellitus, edema or renovascular hypertension were not allowed to participate. Antihypertensive, diuretic and immunosuppressive drugs were withdrawn for at least 4 weeks before enrollment. This study was carried out according to the principles of the Declaration of Helsinki. Healthy subjects and patients gave their informed consent for participation in this protocol, which was approved by the local Medical Ethical Committee.
Each participant collected one 24-hour urine on the day before the renal dextran clearance study. During the in-hospital clearance study subjects received a bolus injection of renal function tracers $^{125}$I-iothalamate and $^{131}$I-hippuran at 7:45 a.m. in the right anticubital vein followed by a constant infusion of these tracers till 15:30 p.m. Following an equilibration period till 10:00 a.m., two clearance periods from 10:00 to 12:00 a.m. and 12:00 to 14:00 p.m. were performed to estimate GFR and ERPF. At 14.00 p.m. an injection of 20 ml dextran-1 (Promiten®, Pharmacia, Uppsala, Sweden) was given to prevent anaphylactic reactions during the subsequent dextran infusion. At 14:15 p.m. a 15-minute infusion of a mixture of 60 mg/kg Rheomacrodex® and 60 mg/kg Macrodex® was administered. After 30 minutes equilibration one clearance period from 15:00 to 15:30 p.m. was performed with bracketed plasma samples to estimate dextran clearances. Throughout the study day patients remained in supine position, except when voiding. Patients were allowed to take their usual breakfast at 8:30 a.m. Thereafter patients received no food, but had to drink at least 250 ml/hr to establish a sufficient diuresis. From 14:00 to 15:00 p.m. patients received an oral water load of 15 ml/kg to promote diuresis for estimation of fractional dextran clearances.

Blood pressure was recorded at 9:00 a.m. with an automated device (Dinamap®) during 15 minutes at 1 minute intervals. Serum and urinary chemistry were determined with an automated multi-analyzer (SMA-C-II®, Technicon Instruments Inc., Tarry Town, NY, USA), whereas urinary protein was determined with the pyrogallol red-molybdate method [53]. GFR and ERPF were calculated according to a previously described method using a constant infusion of $^{125}$I-iothalamate and $^{131}$I-hippuran, respectively [54]. Plasma and urine dextran samples were precipitated with 100% TCA to a final concentration of 20% and supernatants were stored at 4°C. At the next day, dextran clearances were estimated by GPC and anthrone assay, HPLC and anthrone assay, and HPCL and on-line refractometry. This allows a comparison of the three different laboratory methods with respect to absolute values of dextran sieving coefficients in healthy subjects and patients with non-diabetic proteinuria as well as their ability to discriminate on glomerular size selectivity to dextrans between both study groups. Dextran clearances were assessed relative to GFR as estimated by $^{125}$I-iothalamate clearances. Logarithmically transformed fractional dextran clearances were plotted against the molecular radius to compose renal dextran sieving curves over a range from 30 to 60Å. Urinary albumin and IgG excretion, as well as albumin and IgG clearance relative to creatinine clearance, were measured from the 24-hour urine samples according to a previously described method [55]. Glomerular size selectivity to proteins was expressed as the clearance of IgG relative to that of albumin, whereas aselective proteinuria is characterized by an index greater than 0.20 [56].
Figure 1. Effect of plasma precipitation with TCA 20% (panel A), TCA 5% (panel B), and zinc sulfate (panel C) on the elution pattern of dextran detected by anthrone assay (open squares) or on-line refractometry (closed circles). RI = units of on-line refractometry; [dextran] = dextran concentration by anthrone assay.
Data analysis

Data are expressed as mean and SD, unless otherwise indicated. Differences between and within groups were tested with (un)paired student-T test. Statistical significance was assumed at a p-level smaller than 5%.

Results

Sample preparation

Plasma was the most accurately deproteinized using 20% TCA with a protein recovery of only 0.2% (Table 3). Treatment of plasma with 5% TCA or ZnSO4 resulted in a significant higher remaining protein sample concentration of respectively 2.5 and 3.4%. Plasma supernatants which were deproteinized by 5% TCA and ZnSO4 showed a concomitant peak in the dextran elution pattern during on-line refractometry which was not detectable by anthrone assay (Figure 1). Urine was completely deproteinized by 5 and 20% TCA, whereas protein recovery in urine after ZnSO4 precipitation amounted 0.6%. Dextran recovery in plasma and urine supernatants was significantly higher after 20% TCA precipitation compared to 5% TCA and ZnSO4 precipitation (Table 3). However, dextran recovery in plasma supernatant was greater than 100% after 20% TCA precipitation, whereas it was not significantly different from 100% after 5% TCA or zinc sulfate deproteinization. Dextran recovery in urine supernatant was not significantly different from 100% after 20% TCA precipitation, whereas a significant loss of 11% dextrans occurred in urine supernatants after 5% TCA and ZnSO4 precipitation.

We decided to use sample precipitation with 20% TCA to avoid disturbances by proteins in plasma and urine supernatant during on-line refractometry in our further analyses.

Table 3. Remaining relative protein and dextran concentration after deproteinization of plasma and urine samples with 20% TCA, 5% TCA and ZnSO4.

<table>
<thead>
<tr>
<th></th>
<th>Plasma protein</th>
<th>Urine protein</th>
<th>Plasma dextran</th>
<th>Urine dextran</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% TCA</td>
<td>0.2±0.3</td>
<td>0.0</td>
<td>118±9</td>
<td>97±7</td>
</tr>
<tr>
<td>5% TCA</td>
<td>2.5±0.4*</td>
<td>0.0</td>
<td>94±8*</td>
<td>89±6*</td>
</tr>
<tr>
<td>ZnSO4</td>
<td>3.4±0.1*</td>
<td>0.6±0.2*</td>
<td>96±8*</td>
<td>89±10*</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. * p<0.05 versus 20% TCA.

Calibration

The calibration procedure revealed a variability smaller than 5% for GPC and HPLC systems during the study (Table 4). The influence of the molecular weight correction for polydispersity of standard dextrans and of the application of different
Laboratory procedure to measure dextran

relations between $R_{st}$ and molecular weight is depicted in figure 2. Fractional dextran clearances progressively increase over the entire molecular size range if the molecular weight correction would be omitted. A further rightward shift of fractional dextran clearances from 40Å, but not in the low molecular size range, would be induced if the relation between $R_{st}$ and molecular weight according to Oliver et al. [52] would be applied in stead of that according to Granath et al. [46].

Calibration with three different sets of standard dextrans revealed significant differences in fractional dextran clearances (Figure 2). Fractional dextran clearances were lower in the molecular size range from 40 to 60Å in case standard dextrans from Serva were used instead of those from Pharmacia. Dextran sieving coefficients were lower in the molecular size range smaller than 46Å and higher in the molecular size range greater than 50Å in case standard dextrans from Serva were used in stead of standard dextrans with very low polydispersity provided by Dr. Granath. Notably, this latter difference is of a magnitude that is representative of the difference in dextran sieving between healthy subjects and patients with nephrotic range proteinuria.

**Table 4. Variability in calibration at GPC and HPLC during one year using the standard dextrans F8, F40 and F75 (n=9; upper part) and a comparison of calibration with three different sets of standard dextrans at HPLC and anthrone assay (n=4; lower part).**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Slope</th>
<th>CV (%)</th>
<th>Constant</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8/F40/F75</td>
<td>GPC+ant</td>
<td>-0.0112±0.0004</td>
<td>3.4</td>
<td>0.889±0.029</td>
</tr>
<tr>
<td></td>
<td>HPLC+ant</td>
<td>-0.0126±0.0003</td>
<td>2.0</td>
<td>0.845±0.020</td>
</tr>
<tr>
<td></td>
<td>HPLC+RI</td>
<td>-0.0124±0.0004</td>
<td>2.8</td>
<td>0.809±0.040</td>
</tr>
<tr>
<td>F8/F40/F75</td>
<td>HPLC+ant</td>
<td>-0.0120±0.0004</td>
<td>3.5</td>
<td>0.854±0.021</td>
</tr>
<tr>
<td>T10/T40/T70</td>
<td>HPLC+ant</td>
<td>-0.0109±0.0003</td>
<td>2.5</td>
<td>0.822±0.007</td>
</tr>
<tr>
<td>D9/D10/D11/D13</td>
<td>HPLC+ant</td>
<td>-0.0097±0.0003</td>
<td>2.8</td>
<td>0.720±0.013</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. CV = coefficient of variance; ant = anthrone assay; RI = on-line refractometry.

**Variance of dextran assay**

Repetitive assays of a plasma supernatant with dextran by GPC and anthrone assay showed a variance of dextran concentration between 14 and 25% over a range from 30 to 60Å. HPLC and anthrone assay showed a comparable variance in that range (17 to 20%), whereas HPLC and on-line refractometry showed a clearly lower variance of maximally 4% (Figure 3). Repetitive assays of an urine supernatant
Figure 2. Left panel: effect of differences in the calibration procedure on fractional dextran clearances in one healthy subject; Applications used in the present study (closed circles), applications used by Granath et al. [46] without molecular weight correction for polydispersity of standard dextrans (open squares), applications used by Oliver et al. [52] without molecular weight correction for polydispersity of standard dextrans (closed triangles). Right panel: effect of calibration with different sets of standard dextrans on fractional dextran clearances in one healthy subject; F8, F40, and F75 (Serva, Heidelberg, Germany, closed circles); T10, T40, and T70 (Pharmacia, Uppsala, Sweden, open squares); D9, D10, D11, and D13 (Dr. K.A. Granath, Uppsala, Sweden, closed triangles).

with dextran by GPC and anthrone assay showed a variance of dextran concentration from 8 to 18% in the range from 30 to 52Å, whereas it increased up to 43% for dextrans of 60Å. HPLC and anthrone assay showed a slightly higher variance in the range from 30 to 48Å, and much higher variances for dextrans greater than 52Å (up to 78%). HPLC and on-line refractometry revealed the lowest variance of maximally 7% for dextrans from 30 to 48Å, but the variance increased up to 200% for dextrans greater than 56Å. Subsequent calculation of urine-to-plasma dextran concentration index revealed highest variance for GPC and anthrone assay and the lowest variance for HPLC and on-line refractometry in the range from 30 to 48Å. GPC and anthrone assay showed the lowest variance in the range from 54 to 60Å, whereas HPLC and anthrone assay or on-line refractometry showed clearly higher variances.
Figure 3. Variance of plasma dextran concentration (A), urine dextran concentration (B), and urine to plasma index (C) as measured by GPC and anthrone assay (closed bars), HPLC and anthrone assay (open bars), and HPLC and on-line refractometry (cross-hatched bars).
Reproducibility tests on the anthrone assay showed that control samples without dextrans had an extinction coefficient of 0.002±0.008 with a variance higher than 200%. Samples with 0.005 mg/ml dextran showed an extinction coefficient of 0.075±0.014 with a variance of 19%, whereas samples with a dextran concentration from 0.010 to 0.110 mg/ml had a variance of extinction coefficients ranging from 3 to 7%. GPC and HPLC assays revealed dextran concentrations smaller than 0.005 mg/ml from 52 to 60Å in urine supernatant.

**Table 5. Baseline characteristics of healthy subjects and patients with non-diabetic proteinuria.**

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43±12</td>
<td>42±15</td>
</tr>
<tr>
<td>Gender (f/m)</td>
<td>5/10</td>
<td>4/13</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>1.99±0.21</td>
<td>1.94±0.17</td>
</tr>
<tr>
<td>U_protein (g/day)</td>
<td>0</td>
<td>7.8±5.2*</td>
</tr>
<tr>
<td>S_albumin (g/l)</td>
<td>44±3</td>
<td>35±6*</td>
</tr>
<tr>
<td>S_cholesterol (mmol/l)</td>
<td>5.1±0.9</td>
<td>7.2±2.9*</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>126±11</td>
<td>142±27*</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>70±8</td>
<td>81±14*</td>
</tr>
<tr>
<td>GFR (ml/min/1.73m²)</td>
<td>107±17</td>
<td>76±23*</td>
</tr>
<tr>
<td>ERPF (ml/min/1.73m²)</td>
<td>447±81</td>
<td>392±207</td>
</tr>
<tr>
<td>FF (%)</td>
<td>24±2</td>
<td>21±4*</td>
</tr>
<tr>
<td>U_albuminE (µg/min)</td>
<td>5±4</td>
<td>3465±3588*</td>
</tr>
<tr>
<td>U IgG E (µg/min)</td>
<td>2±1</td>
<td>156±202*</td>
</tr>
<tr>
<td>Cl_albumin (10⁻⁵)</td>
<td>8±4</td>
<td>1443±2048*</td>
</tr>
<tr>
<td>Cl IgG (10⁻⁵)</td>
<td>11±5</td>
<td>194±177*</td>
</tr>
<tr>
<td>IgG/albumin index</td>
<td>-</td>
<td>0.16±0.06</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. Abbreviations are: f = female; m = male; BSA = body surface area; U_protein = proteinuria; S_albumin = serum albumin; S_cholesterol = serum cholesterol; U_albuminE = urinary albumin excretion; U IgG E = urinary IgG excretion; Cl_albumin = fractional albumin clearance; Cl IgG = fractional IgG clearance. * p<0.05 versus healthy subjects.

**Dextran clearance studies**

Healthy subjects and patients showed comparable age, gender distribution, and body surface area. Patients had proteinuria of 7.8±5.2 g/day with an IgG to albumin clearance index of 0.16 (range: 0.07 to 0.25). Only 5 of the 17 patients showed an IgG to albumin clearance index greater than 0.20. Patients demonstrated higher systolic and diastolic blood pressure, and serum cholesterol, but a lower GFR, FF, and serum albumin than healthy subjects (Table 5). Neither GFR, ERPF, or filtration fraction changed significantly from baseline values during dextran
infusion in healthy subjects or patients. The dextran sieving coefficients within both groups depended on the used dextran assay. GPC and anthrone assay showed significant higher dextran sieving coefficients than HPLC and anthrone assay from 30 to 54Å in healthy subjects and from 42 to 58Å in patients (Figure 4). GPC and anthrone assay revealed lower dextran sieving coefficients than HPLC and anthrone assay from 30 to 36Å in patients. HPLC and on-line refractometry revealed significant lower dextran sieving coefficients than HPLC and anthrone assay from 36-54Å in healthy subjects and from 36-52Å in patients. 

The dextran assays also differed with respect to their ability to discriminate on glomerular size selectivity to dextrans between healthy subjects and patients with non-diabetic proteinuria. GPC and anthrone assay revealed the well-known differences in dextran sieving (Figure 5). Patients have higher dextran sieving coefficients from 58 to 60Å and lower dextran sieving coefficients from 30 to 40Å. In contrast, HPLC and anthrone assay elicited no significant differences between dextran sieving in both groups, although patients showed a trend of increased dextran sieving coefficients for dextrans larger than 50Å. HPLC and on-line refractometry revealed significant higher dextran sieving coefficients from 46 to 52Å in patients, but it did not show significant differences in the low molecular weight dextran range as well as in the range from 54 to 60Å. Irrespective of the performed dextran assay, the fractional clearance of dextran 56Å was not significantly related with the fractional IgG clearance or the IgG to albumin index in our patients.

Discussion

The present study is the first to show that considerable quantitative and qualitative variations in fractional dextran clearances are generated when one varies the different available laboratory methods with respect to sample preparation, calibration, size-exclusion chromatography and dextran detection. The standard and most frequently used laboratory assay to measure fractional dextran clearances consists of size-exclusion chromatography by GPC, and measurement of dextran concentration in eluted fractions by the anthrone assay [46-47]. This procedure is known to be complex, time-consuming, elaborate, and susceptible to interfering factors such as changes in flow rate, variability of calibration, errors in collection of eluted fractions, or disturbances in dextran detection. Because of that, HPLC and on-line refractometry have been introduced as alternative methods of size-exclusion chromatography and dextran detection [50]. HPLC assays are of shorter duration, allow use of smaller samples, and include control of flow rate and pressure. On-line refractometry prevents variability due to errors in fraction collection and enables more sensitive detection of dextrans greater than 65Å [19-20,45]. Despite the mentioned advantages of HPLC and on-line refractometry, some doubts may arise upon their performance. Thomas et al. [31,42] using HPLC and on-line refractometry failed to detect increased fractional clearances of dextrans greater than 50Å in patients with membranous glomerulopathy and nephrotic proteinuria, whereas
fractional clearances of dextrans greater than 50Å were increased in patients with thin membrane nephropathy without proteinuria. Wiegmann et al. [32] using HPLC and on-line refractometry observed a flow through large aselective pores in diabetic patients with micro-albuminuria which is comparable to that in diabetic patients with nephrotic range proteinuria [7,10,12,13,17,21,25]. A previous non-published study from our own department using HPLC and anthrone assay failed to detect improvement of glomerular size selectivity by dextran sieving during antiproteinuric treatment with ACE inhibition, while glomerular size selectivity to proteins significantly improved [26]. Remarkably, no analysis has been published so far that compared the new techniques with the standard techniques on quantitative and qualitative aspects of measurement of fractional dextran clearances.

![Figure 4. Dextran sieving curves of healthy subjects (A) and patients with non-diabetic proteinuria (B) as measured by GPC and anthrone assay (closed squares), HPLC and anthrone assay (open squares), and HPLC and on-line refractometry (closed triangles). *p<0.05 GPC and anthrone assay versus HPLC and anthrone assay; #p<0.05 HPLC and anthrone assay versus HPLC and on-line refractometry.](image)

The first aspect of the present study involved methods of dextran sample preparation. Initially, dextran samples were not specifically prepared for size-exclusion chromatography or no information has been presented on the method of
Laboratory procedure to measure dextran

Sample preparation. In later studies, dextran samples were prepared before assay by precipitation of proteins to prevent damage of polymer gels and interference of dextran detection by proteins. The most applied agents for protein precipitation of dextran samples are trichloroacetic acid in varying concentration and zinc sulfate. We observed that complete deproteinization of plasma was only achieved if TCA was used to a final concentration of 20%, whereas a small amount of proteins were recovered from plasma supernatant after 5% TCA and zinc sulfate deproteinization. Zinc sulfate deproteinization also revealed small protein recovery in urine supernatant. Incomplete protein precipitation is not of great importance in case dextran concentration is measured by the anthrone assay, since this assay is not affected by proteins [48]. In contrast, a small amount of protein in supernatant clearly interfered with the dextran elution profile during on-line refractometry. One previous study showed no confounding peaks during HPLC and on-line refractometry when dextran samples were precipitated by picric acid [49]. After precipitation with picric acid, dextran is recovered from supernatant by precipitation with nine volumes ethanol, lyophilizing, and reconstituting in eluens. This revealed a small, but significant fractionation of dextran as well as 10% loss of dextrans with low molecular weight [49]. No data are published so far on the influence of TCA or zinc sulfate on dextran recovery. We observed that dextran recovery in plasma supernatant was higher than 100% by 20% TCA precipitation and dextran recovery in urine supernatant was less than 100% by 5% TCA and zinc sulfate precipitation. The consequence for dextran sieving coefficients seems to be limited, since urine to plasma dextran concentration index over the entire molecular size range is comparable for each of the protein precipitation methods. Altogether, it is of great importance to choose a precipitation method which completely deproteinizes samples without co-precipitation of dextrans, or to choose a dextran detection method which is not disturbed by proteins in case of incomplete protein precipitation. Sample preparation by 20% TCA precipitation is the method of choice in case of dextran detection by anthrone assay or on-line refractometry, although precipitation by picric acid may be an alternative method in case of on-line refractometry.

We secondly studied the variability of calibration of GPC and HPLC systems by fractionation of standard dextrans with known molecular weight distribution and polydispersity. It is known that macromolecules like proteins do not reveal accurate calibration for size-exclusion chromatography of dextran [50]. To avoid major errors in size-exclusion chromatography of dextran over a long period, it is essential to perform a frequent and uniform calibration [47]. The variability of calibration at GPC and HPLC in the present study reflects an optimal and comparable accuracy of size-exclusion chromatography. However, the absolute values of fractional dextran clearances significantly alter through molecular weight correction for polydispersity of standard dextrans or application of different relations between $R_{st}$ and molecular weight of standard dextrans in the calibration procedure. The molecular weight correction induces lower absolute fractional dextran clearances over the molecular size range from 30 to 60Å in the present study compared to most previous studies. The impact of this correction will be greater in case more polydisperse standard
Dextrans are used for calibration. Despite this molecular weight correction however, differences in polydispersity of standard dextrans still revealed serious changes of fractional dextran clearances in relation to its molecular size as shown by the comparison of calibration with three different sets of standard dextrans.

Figure 5. Dextran sieving curves of healthy subjects (open circles) and patients with non-diabetic proteinuria (closed squares) as measured by GPC and anthrone assay (A), HPLC and anthrone assay (B), and HPLC and on-line refractometry (C). *p<0.05 patients with non-diabetic proteinuria versus healthy subjects.

These differences in fractional dextran clearances are certainly not a chromatographic or detection error since the calibrations were performed on a stable HPLC system with the regular anthrone assay. Altogether, one should consider that the use of sets of standard dextrans with differences in polydispersity for calibration induce differences in dextran sieving coefficients. Size-exclusive chromatographic systems have therefore to be frequently and uniformly calibrated with one set of standard dextrans preferably with a low polydispersity (<1.1). An alternative approach is to calibrate with only one sample of a dextran with known broad molecular mass distribution [57]. This method requires no software to calculate calibration regression lines and is not affected by limited number and spread of calibration dextrans and could therefore be more suitable for routine laboratories.
Thirdly, we compared the variability of the GPC and anthrone assay, HPLC and anthrone assay and HPLC and on-line refractometry. So far, no direct comparison of the assays has been published previously. The GPC and anthrone assay revealed an intra-assay variance of dextran sieving coefficients between 10 and 20% in the range from 30 to 50Å and a variance up to 100% for dextrans greater than 50Å [56]. These high variances in the high molecular weight range was explained by a lack of sensitivity to detect low dextran concentrations by the anthrone assay. Interstudy variance of dextran sieving coefficients in healthy subjects amounted between 6.4% for 30Å to and 2.7 to 3.8% for the remaining fractions by GPC and anthrone assay [33]. HPLC and on-line refractometry revealed variances lower than 10% [43,45]. With respect to the present comparison, it could be expected that GPC is more sensitive to variance than HPLC, since GPC last longer and are less well controlled with respect to flow and temperature conditions than HPLC. Furthermore, the anthrone assay could be less accurate in detection of low dextran concentrations, whereas on-line refractometry may be affected by non-precipitated proteins. In the present analysis, HPLC is indeed superior to GPC with respect to variance of urine-to-plasma dextran concentration in the range from 30 to 48Å, whereas on-line refractometry is indeed superior to the anthrone assay. However in the range from 54 to 60Å, GPC is superior to HPLC independent of the method of dextran detection. The high variances of dextran concentrations from 54 to 60Å are due to the poor sensitivity of dextran detection by anthrone assay as well as on-line refractometry in that range. This may imply that only large differences in glomerular size selectivity to dextrans or smaller differences in a large number of patients can be detected by the tested dextran assays. In this respect, differences in glomerular size selectivity between healthy subjects and patients with proteinuria greater than 3 gram/day as well as changes in glomerular size selectivity during considerable reduction of proteinuria have been detected.

We fourth measured fractional dextran clearances in healthy subjects and patients with non-diabetic proteinuria with each of the three dextran assays. One might expect that the dextran sieving coefficients in a patient are independent of the assay which has been used. We however observed significant differences between GPC and HPLC with anthrone assay or on-line refractometry in both study groups. Except for those quantitative differences, the dextran assays also showed qualitative differences in the comparison between healthy subjects and patients with proteinuria. Only GPC and anthrone assay revealed differences in dextran sieving between both groups which are in agreement with previous studies on this issue: patients with proteinuria showed increased dextran sieving coefficients from 58 to 60Å and decreased dextran sieving coefficients from 30 to 40Å. HPLC and anthrone assay tended to reveal increased dextran sieving coefficients from 50 to 60Å, but this difference failed to reach statistical significance. HPLC and on-line refractometry revealed significantly increased dextran sieving coefficients from 46 to 52Å in patients. In contrast with previous studies [20,23,45], HPLC and anthrone or on-line refractometry failed to detect differences in the low molecular weight range between healthy subjects and patients with proteinuria. The important question is which of our
tested assays represents the true state of glomerular size selectivity. Considering
the presence of rather selective proteinuria in our patients, it may have been
expected that dextran sieving coefficients from 54 to 60Å are comparable in both
groups, whereas fractional clearances of dextrans smaller than 40Å are decreased
in patients with proteinuria. None of the three assays revealed such a dextran
sieving pattern. On the one hand, GPC and anthrone assay showed the lowest
variance of urine-to-plasma dextran concentration for dextrans greater than 54Å and
revealed significant higher fractional clearances of dextrans in that range. HPLC and
on-line refractometry which showed the lowest variance from 30-48Å revealed
increased fractional clearances of dextrans from 46-52Å. On the other hand, GPC
and anthrone assay reveals the highest variance of urine-to-plasma dextran
concentrations in the low molecular size range, but it is the only assay which reveals
the expected lower dextran clearances from 30 to 40Å in patients with proteinuria.
We suggest that HPLC is inferior to GPC with respect to size-exclusion
chromatography of dextrans because of differences in gel characteristics or by
pressure-induced differences in retention, since we can exclude other factors like
differences in sample preparation and conservation, calibration, or dextran
detection. It appears that GPC and anthrone assay is the most sensitive assay to
detect impaired glomerular size selectivity by fractional dextran clearances despite
its high variance in the range of interest. That neither of the dextran assays reveals
a significant relation between fractional dextran clearance and fractional IgG
clearance or IgG to albumin index may be explained by the characteristics of our
patients as well as by the limitations of protein clearances and -indices for
measurement of glomerular size selectivity.

It has to be considered that differences in clearance protocols may well have
a role in differences between studies with respect to fractional dextran clearances.
First, different tracers for estimation of GFR have been used. Radioactive labeled
iothalamate does not interfere with dextran detection. In contrast, inulin is detected
by the anthrone assay [48]. Although it does not adversely affect fractional
clearances of high molecular weight dextrans, fractional clearances of small
dextrans may thus be overestimated by simultaneous detection of inulin and dextran
[56]. Second, dextran has to be infused in small concentrations to prevent changes
in renal hemodynamics. The recommended dosage of dextran indeed did not change
GFR or ERPF from baseline values in the present study in accordance with data
from Mogensen et al. [5]. Third, differences in fractional dextran clearances are
induced by different methods of infusion [49]. It has been recommended to use bolus
and continuous infusion of dextran to avoid changes in plasma concentration of
small dextrans due to their rapid renal clearance. Bolus infusion without continuous
infusion of small dextrans reveals lower fractional dextran clearances in the low
molecular weight range. We indeed showed lower fractional clearances in that range
in both our groups as compared to previous studies which used bolus and
continuous infusion.

In conclusion, differences in laboratory methods induce quantitative and
qualitative differences in fractional dextran clearances. We prefer 20% TCA sample
deproteinization, a frequent and uniform calibration procedure with one set of calibration dextrans with low polydispersity, size-exclusion chromatography by GPC, and dextran detection by anthrone assay. Since use of this laboratory procedure still reveal a high variance in dextran sieving coefficients in the important pore size range from 54 to 60Å, it may well be that only major differences in glomerular size selectivity are detected. Concentration of dextran samples or improvement of dextran detection techniques would be helpful in gaining more sensitivity to measure changes in glomerular size selectivity by fractional dextran clearances.

Acknowledgments

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