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Short communication

Determination of inulin in plasma and urine by reversed-phase high-performance liquid chromatography

R. Dall'Amico*, G. Montini, L. Pisanello, G. Piovesan, S. Bottaro, A.T. Cracco, G. Zacchello, F. Zacchello

Department of Pediatrics, University of Padua, Via Giustiniani 3, 35128, Padua, Italy

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Abstract

We report a new HPLC procedure for measuring inulin in plasma and urine. Samples after dilution are boiled in mild acidic conditions and then analyzed on a C_{18} column. Solvent system A is 3.2 m*M* HCl, pH 2.5, and B is acetonitrile–3.2 m*M* HCl (60:40, v/v), pH 2.5. The separation is carried out in 8 min with a flow-rate of 1.0 ml/min and the absorbance monitored at 280 nm. The relationship between inulin and the recorded peak area is linear from 0.2 to 3.2 mg/ml with a correlation coefficient of 0.999 for plasma and 0.999 for urine. Within-run precision, measured at three inulin concentrations, ranged from 0.9 to 1.7% in plasma and from 0.8 to 1.2.% in urine. Between-run precision varied in plasma from 2.7 to 3.2.% and in urine from 3.0 to 3.3%. Analytical recovery ranged from 102 to 107% in plasma and from 101 to 105% in urine, respectively. The method is sensitive, selective and only 30-μl samples are required. Therefore, it could be used to evaluate the glomerular filtration rate even in small babies and to perform studies in animals.

1. Introduction

The assessment of glomerular filtration rate (GFR) is the most important parameter used to evaluate the renal function and its response to treatments in patients with kidney diseases [1,2]. Inulin clearance (C_{In}) is considered as the reference method for the evaluation of GFR. Inulin meets all the requirements for an excellent tracer for GFR measurement: it is non-toxic, freely filterable at the glomerulus, neither reabsorbed nor secreted by the tubules and not bound by plasma proteins [3,4]. However, despite these characteristics, C_{In} is not commonly used in clinical practice because the measurement of inulin is often difficult and inaccurate.

Most of the methods for the determination of inulin are based on reactions with the fructose derived from acid hydrolysis. Several substances such as resorcinol, anthrone, diphenylamine, and indole-3-acetic acid, have been used and the colour formed from the reaction with fructose measured photometrically. These colourimetric assays are often inaccurate, mainly due to interference with other plasmatic hexoses, and potentially dangerous, owing to the use of concentrated corrosive reagents [5–8]. Other procedures, involving the use of enzyme treatment of inulin after acid hydrolysis, or the application of

* Corresponding author.

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HPLC, are more sensitive and specific but time-consuming [9–12].

We describe here a rapid and sensitive reversed-phase HPLC procedure for the measurement of inulin in plasma and urine after a simplified acid hydrolysis of the sample.

2. Experimental

2.1. Chemicals

Acetonitrile (LiChrosolv) and hydrochloric acid (Suprapur) were purchased from Merck (Darmstadt, Germany). Water was obtained from a Milli-Q Waters purification system (Millipore, Milford, MA, USA). All solutions were passed through a 0.45-μm Millipore filter and degassed before use. Inutest, which is a polyfructosan, was obtained from Laevosan (Linz, Austria). The other chemicals used were analytical grade.

2.2. Samples

Blood and urine samples were obtained from six children aged 3 to 10 years, affected by different nephropaties, and from adult voluntaries with normal function.

2.3. Standard solutions

A stock solution of 64 mg/ml inulin and a series of dilutions (4, 8, 16, 32 mg/ml) were prepared in water. Standard solutions of 50 μl were added to 0.95 ml of pooled plasma and urine to obtain a concentration range of 0.2–3.2 mg/ml.

2.4. HPLC apparatus

The liquid chromatograph consisted of two pumps Model 510 (Waters Assoc., Milford, MA, USA), a detector Model 430, an autosampler Model 465, and a chromatography data system, Model 450-MT (Kontron Instruments, Milan, Italy).

2.5. Sample preparation for HPLC

Aliquots of 30 μl of plasma and urine were diluted 1:10 and 1:100 (v/v), respectively, with saline solution; 200-μl aliquots were mixed for 30 s with 100 μl of 70% $HClO_4$, centrifuged 5 min at 13.200 *g* and boiled for 10 min to hydrolyze inulin to fructose and to convert fructose to hydroxymethylfuraldehyde (HMF). The samples were cooled on ice for 5 min. Aliquots of 10 μl were analyzed directly on HPLC.

2.6. HPLC analysis

A LiChrospher 100 RP-18 column (125 × 4 mm I.D.), 5 μm particle size (Merck) was used. Solvent system A was 3.2 m*M* HCl, pH 2.5, and B was acetonitrile–3.2 m*M* HCl (60:40, v/v), pH 2.5. The chromatographic separations were carried out at room temperature in 5 min with a gradient of 0 to 20% B and 3 min with A–B (80:20, v/v). The flow-rate was 1.0 ml/min. The absorbance was monitored at 280 nm.

3. Results

Overlapped chromatograms of plasma (Fig. 1b) and urine (Fig. 1c) samples before and after inulin addition are compared with a chromatogram of an aqueous standard solution (Fig. 1a) of inulin (0.1 mg/ml). The chromatographic profiles of both plasma and urine samples do not show any interfering substance at the retention time corresponding to the recorded peak.

The HMF peak is well resolved with a retention time of 4.82 ± 0.11 min ($n = 50$) with plasma and 4.86 ± 0.10 min ($n = 50$) with urine, respectively.

3.1. Linearity

The linearity of the method was evaluated for plasma and urine. The calibration curve shows a linear relationship between the peak areas and inulin concentration over a wide range of concentrations (0.2, 0.4, 0.8, 1.6 and 3.2 mg/ml). Each point was established from an average of 10

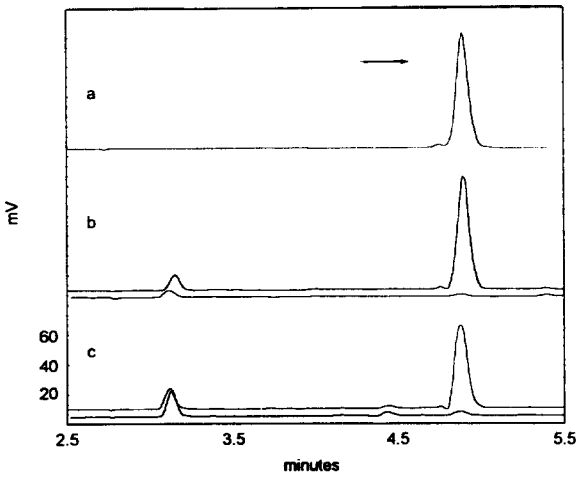

Fig 1. Representative chromatograms of (a) an inulin (0.1 mg/ml) standard solution compared with overlapped chromatograms of (b) plasma and (c) urine samples collected before and after inulin administration. The peak corresponding with the inulin product HMF is marked with an arrow.

determinations. The regression equation of the curve obtained from plasma samples was $y = 0.0734x + 0.1283$, were y is the inulin concentration (mg/ml) and x is the peak area. The standard error of the slope was 0.00178 and the correlation coefficient (r) was 0.999. The regression equation of the curve obtained analyzing urine samples was $y = 1.0669x + 1.1653$. The standard error of the slope was 0.016 and the correlation coefficient (r) was 0.999. The signal-to-noise ratio at an inulin concentration of 0.1 mg/ml was 10, both in plasma and urine.

3.2. Precision

Within- and between-run precision data, calculated from 10 replicate analyses of 3 urine and 3 plasma samples with low, medium and high inulin contents are reported in Table 1.

3.3. Analytical recovery

Recovery of inulin from supplemented plasma varied from 102 to 107% over a concentration range of 0.2–3.2 mg/ml; recovery from urine was 101–105% over a concentration range of 0.8–3.2 mg/ml (Table 2).

3.4. Interferences

Glucose and the following co-administered drugs, usually taken by patients with kidney diseases, did not interfere with the assay: calcitriol, corticosteroids, cyclosporine, azathioprine, nifedipine, atenolol, calcium carbonate, erythropoietin.

Table 1
Precision of the method in plasma and urine

Within-run precision ($n = 10$)			Between-run precision ($n = 10$)		
Inulin concentration (mg/ml)		C.V. (%)	Inulin concentration (mg/ml)		C.V. (%)
Added	Found (mean ± S.D.)		Added	Found (mean ± S.D.)	
Plasma					
0.2	$0.207 \pm 0.3 \cdot 10^{-2}$	1.7	0.2	$0.202 \pm 0.5 \cdot 10^{-2}$	2.7
0.8	$0.811 \pm 1.0 \cdot 10^{-2}$	1.2	0.8	$0.817 \pm 2.5 \cdot 10^{-2}$	3.1
1.6	$1.624 \pm 1.2 \cdot 10^{-2}$	0.9	1.6	$1.615 \pm 5.1 \cdot 10^{-2}$	3.2
Urine					
0.8	$0.802 \pm 0.9 \cdot 10^{-2}$	1.2	0.8	$0.794 \pm 0.2 \cdot 10^{-2}$	3.2
1.6	$1.614 \pm 1.7 \cdot 10^{-2}$	1.1	1.6	$1.613 \pm 5.4 \cdot 10^{-2}$	3.3
3.2	$3.228 \pm 2.5 \cdot 10^{-2}$	0.8	3.2	$3.213 \pm 9.7 \cdot 10^{-2}$	3.0

Table 2
Analytical recovery of inulin from plasma and urine

Inulin concentration (mg/ml)		Recovery (%)
Added	Found (mean ± S.D.)	
Plasma (n = 20)		
0.2	$0.210 \pm 0.5 \cdot 10^{-2}$	105
0.8	$0.863 \pm 2.6 \cdot 10^{-2}$	107
1.6	$1.634 \pm 5.2 \cdot 10^{-2}$	102
Urine (n = 20)		
0.8	$0.808 \pm 2.6 \cdot 10^{-2}$	101
1.6	$1.632 \pm 5.3 \cdot 10^{-2}$	102
3.2	$3.360 \pm 9.9 \cdot 10^{-2}$	105

4. Discussion

C_{In} is considered to be the "gold standard" for both clinical and investigative assessment of GFR. However, the values obtained for clearance, particularly when a single injection technique is used, may depend on hydration of the patients, dose of inulin, timing and number of blood samples, pharmacokinetic model, and method used for measuring inulin levels [13]. Several authors have reported that the common techniques for inulin determination are often inaccurate, comporting errors, sometimes relevant, to consider when a C_{In} is carried out [14–17].

Colourimetric assays are commonly used for measuring inulin. Fructose is derived from inulin by acid or enzymatic hydrolysis. It is then reacted with a substrate to produce a coloured reaction product that can be measured photometrically. Despite differences in the substrates used, these methods overestimate inulin in plasma because glucose, structurally similar to fructose, forms a chromogenic compound. Several techniques have been used to reduce these interferences: removal of glucose by yeast or autoxidation, measurement of inulin after precipitation or solid-phase extraction, and modification of reaction conditions [8]. However, all these additional steps are time-consuming and sometimes a source of additional errors in the procedure.

Recently, complete enzymatic assays have been reported [9–11]. These are more sensitive than the colourimetric methods and require no corrosive reagents. However, even in the enzymatic methods physiological glucose concentrations in plasma cause a considerable background signal that reduces the reproducibility and the linearity of the procedures. Enzymatic glucose oxidation has been proposed recently by Delanghe et al. to minimize this interference [18].

It is well known that hexoses heated in acid solution form hydroxymethylfuraldehyde. An analogous reaction is carried out in the method described in this paper: inulin is hydrolyzed to fructose and then converted to HMF in acid solution at high temperature. This preparative step is simple and many samples can be carried out at the same time in less than 30 min for the determination by HPLC.

The method is very sensitive and 30 μl of plasma are sufficient for the assay. This is very important when GFR has to be monitored in infants and young children. Drugs usually administered to patients with renal failure did not interfere with the assay. The cost of the procedure is low and the time needed very short, particulary when an autosampler is available.

In conclusion, this technique is sensitive, easily automated and it could represent a useful tool for measuring inulin in biological fluids.

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