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Rapid and simple determination of inulin in biological fluids by high-performance liquid chromatography with light-scattering detection

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Abstract

We report a new high-performance liquid chromatography method developed for measuring inulin in plasma and urine using ion moderated partition chromatography and evaporative light-scattering detection. Samples are deproteinized with a zinc acetate and phosphotungstic acid solution and added with melezitose as an internal standard. The chromatographic separation is carried out in 16 min at a flow-rate of 0.6 ml/min using deionized water as the mobile phase. Within-run precision, measured at four different concentrations (0.050 mg/ml, 0.150 mg/ml, 0.300 mg/ml and 1.200 mg/ml), ranges from 1.7 to 3.4% in plasma and from 1.5 to 3.5% in urine. Similarly, between-run precision is in plasma from 2.0 to 4.3% and in urine from 2.0 to 4.4%. Analytical recovery ranges from 97.9 to 100.1% in plasma and from 99.1 to 99.7% in urine, respectively. Detection limit (signal-to-noise ratio=3) is 5 μg/ml both in plasma and urine. The method is simple, sensitive, without interference due to hexoses or drugs commonly taken by patients with renal diseases, and offers the advantage of measuring inulin without previous hydrolysis of the molecule.

Keywords: Inulin

1. Introduction

Inulin is a polyfructosan with a molecular weight of 5000 D, that represents an ideal compound for the determination of glomerular filtration rate (GFR) since it is non toxic, not bound by plasma proteins, freely filtered through the glomerulus and eliminated by the kidney without reabsorption or secretion by the renal tubular cells [1–4].

While its clearance, performed by constant infusion (CIn), provides the most accurate method for measuring GFR, it is not routinely used in clinical practice since it is difficult to perform and requires multiple blood and urine samples. However, when an appropriate mathematical model is used, a simple and fast single-injection CIn can be used to measure GFR with accuracy comparable to that obtained by infusion.

Most of the methods for the determination of

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inulin are based on acid hydrolysis of inulin and the reaction of fructose with several compounds such as resorcinol, diphenylamine, anthrone and indole-3-acetic acid that produce a photometric reaction. These methods have the advantage of being simple and inexpensive; however, inaccuracy may often derive from interfering compounds, such as hexoses, with an overestimation of the inulin content [5–7]. Other enzymatic procedures, in which inulin is enzymatically hydrolyzed to fructose [8–12] or converted to sorbitol by sorbitol dehydrogenase [13–15], have been developed to overcome these interferences. Unfortunately, physiological concentrations of glucose may cause a considerable background that reduces the reproducibility and the linearity of the procedure, in particular at low inulin concentrations. Additional steps, such as removal of glucose by yeast, enzymatic [16] or auto-oxidation, measurement of inulin after precipitation, have been proposed to reduce these interferences with the disadvantage of increasing analysis time.

In an attempt of improving analytical reliability several high-performance liquid chromatography (HPLC) methods using reverse phase [17] or electrochemical detection [18,19] have been published in the last years. Yet, despite the improved sensitivity and precision, most of them require long preparation of the sample, including acid hydrolysis or solid-phase extraction of specimens, and high sample volumes.

In this paper we describe a new, rapid and sensitive method for the direct determination of inulin, without previous hydrolysis of the sample, in which partition chromatography with light-scattering detection is used.

2. Material and methods

2.1. Chemicals

Inulin (Inutest) and melezitose were obtained from Laevosan (Linz, Austria) and Fluka (Buchs, Switzerland), respectively. Acetic acid, hydrochloric acid, 5-sulfosalicylic acid, trichlorocetic acid, lead nitrate, zinc acetate dihydrate and phosphotungstic acid 24-hydrate were provided by Merck (Darmstadt, Germany). HPLC grade acetonitrile, acetone, and methanol were from Carlo Erba (Milan, Italy). Micro-Spin Centrifuge Filters Nylon 66, 0.2 μm, were supplied by Alltech Assoc. (Deerfield, IL, USA). Water was obtained from a Milli-Q Waters purification system (Millipore, Milford, MA, USA).

2.2. Patients and inulin clearance

Thirty-one patients (11 males and 20 females) with a median age of 15.6 years (5–30 years) and a body weight from 18 to 82 kg were enrolled in this study. Patients were affected by different nephropathies and underwent an inulin clearance for clinical reasons.

Clearance of inulin was determined according to a standard method during water diuresis and spontaneous micturation. Patients initially received a priming dose of 1 ml/kg of a 10% inulin solution. This was followed by a continuous infusion to obtain plasma levels of about 0.3 mg/ml. The continuous infusion rate was obtained with the following formula:

$$\frac{\text{GFR} \times 25\ \text{mg/dl (inulin concentration)} \times 210\ \text{min (time)}}{\text{inulin concentration (100 mg/ml)}} = \text{volume of inulin solution}$$

where

$$\text{GFR} = \frac{\text{height (cm)} \times 40}{\text{Creatinine}} \times \frac{1.73\ \text{m}^2}{\text{Body surface}}$$

Diuresis was maintained by an oral water intake of 20 ml/kg during the first hour and of 10 ml/kg in the following hours.

After 1 h of equilibration blood and urine samples were collected every 45 min for 3 h by spontaneous micturation and inulin analyzed using the proposed method. GFR was then calculated according to the formula, Clearance = (UV/P) where U and P are the inulin concentration in urine and plasma respectively and V the urine flow-rate.

Four patients (2 male and 2 female) with a median age of 2 years and a body weight from 12 to 19 kg underwent an inulin clearance after single iv injection of 100 mg/kg of the tracer. Blood samples were collected before administration and after 15, 30, 60, 120 and 180 min. GFR has been calculated as reported by Dalton et al. [1]. GFR has been also

evaluated with the creatinine clearance using the Formula of Schwartz.

Plasma and urine samples were also obtained from children and adults with normal renal function and chronic renal failure.

2.3. Solutions

A stock solution of inulin (2.4 mg/ml) and a series of dilutions (0.05–1.2 mg/ml) were prepared in water. An internal standard solution of melezitose (0.5 mg/ml) was also prepared in water. All solutions were stable at 4°C for at least 1 month.

A deproteinizing solution was obtained dissolving 9.1 g of zinc acetate dihydrate, 5.46 g of phosphotungstic acid 24-hydrate and 5.81 ml of acetic acid in water, making up to 100 ml.

2.4. HPLC apparatus

The liquid chromatography consisted of a Model 510 pump, temperature control module and column heater from Waters Assoc. (Milford, MA, USA), a Model 465 autosampler and Model 450-MT chromatography data system from Kontron Instruments (Milan, Italy). The Varex MKIII Evaporative Light Scattering Detector was from Alltech Assoc.

2.5. Sample preparation

One hundred microliters of plasma or 40 fold diluted urine were Vortex-mixed with 30 μl of precipitating reagent and placed in a Branson 2200 ultrasonic bath for 5 min. To the samples 50 μl of internal standard solution was added, and subsequently centrifuged at 13000 *g* for 5 min in a Biofuge A (Heraeus). The supernatant was filtered throughout a 0.2 μm Micro-Spin centrifuge Nylon 66 filters, and 10 μl aliquots analyzed directly by HPLC.

2.6. HPLC analysis

The column used was an Aminex HPX87P (300 mm×7.8 mm ID), 9 μm particle size equipped with a Bio-Rad Carbohydrate Deashing System guard column (a cartridge, 30×4.6 mm, packed with a cation-exchange resin in the hydrogen form and a cartridge, 30×4.6 mm, packed with an anion-exchange resin in the carbonate form) (Bio-Rad Lab., Hercules, CA, USA). The chromatographic separation was carried out in 16 min, using HPLC grade water as mobile phase at a flow-rate of 0.6 ml/min keeping the column temperature at 85°C. The eluent was filtered through a 0.45 (μm Millipore filter and degassed with helium before use. After optimization of the parameters for Varex MKIII ELSD, the drift tube temperature was set at 130°C and the carrier gas flow (air) set at 3.5 slpm (standard litre per min). Output signal attenuation and time constant of recorder were 1/32 and 0.2 s, respectively.

3. Results

3.1. Optimization of the method

The first aim of the study was to set up a simple purification procedure of the samples. Several organic acid solvents such as methanol, acetone, ethanol and acetonitrile were tested. Later they were excluded because of the low solubility of inulin at high solvent concentration. Other acid compounds including trichloroacetic acid, sulfosalicilyc acid and hydrochloric acid were also excluded since they may produce a hydrolysis of the polyfructosan chain. Ultimately a deproteinizing solution containing phosphotungstic acid and zinc acetate, mainly used in the evaluation of carbohydrate content in dairy products, was used [20].

Several variables to optimize the separation of inulin peak from that of other endogenous compounds were tested. We initially used a polymeric column packed with sulphonic ion-exchanger in the calcium form proposed by other authors for measuring inulin in plasma and urine [18,19]. However, a scanty resolution of the inulin peak was observed in the chromatogram. In addition, the presence of ionic compounds not retained in the precolumn may produce an abnormal nebulization of the mobile phase and a disturbance of the baseline as a consequence.

The chromatogram obtained analyzing plasma samples with the proposed method are shown in Fig. 1. The polymeric column in the Pb form allows a better identification of the inulin peak, and the pre-

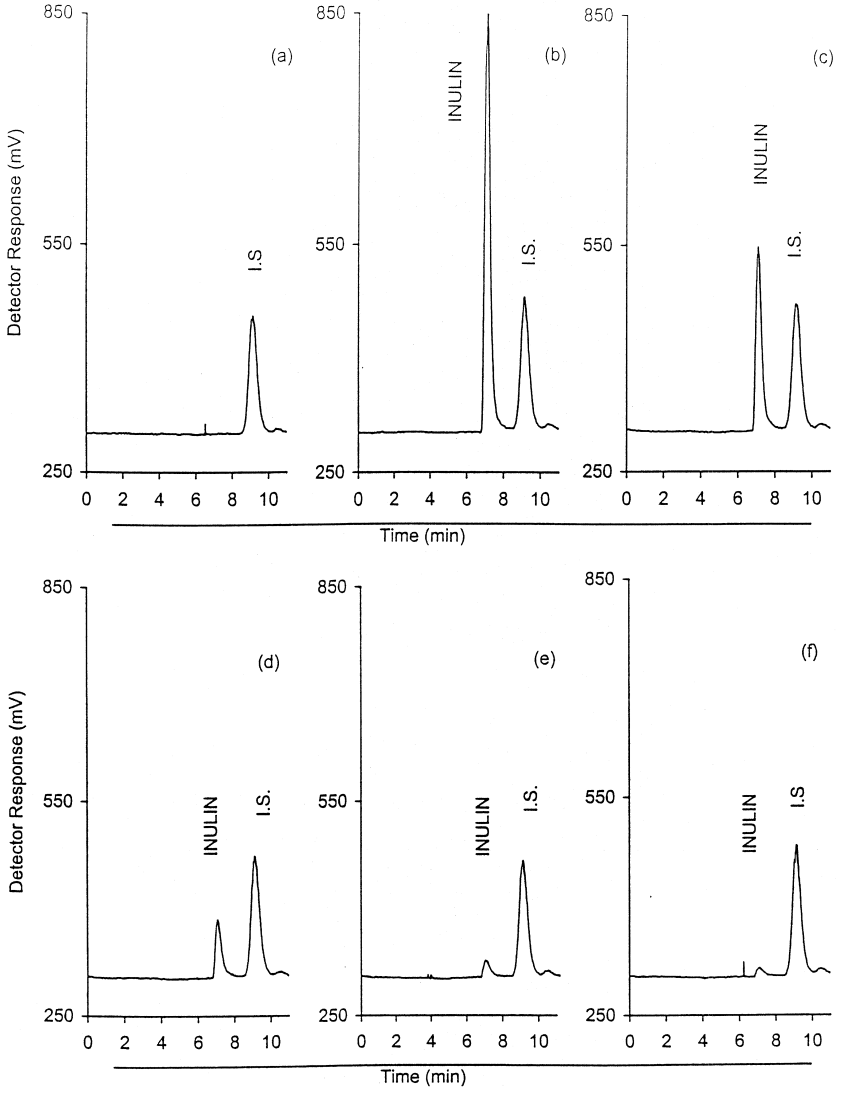

Fig. 1. Chromatographic profile of plasma from a patient who underwent a single injection. Inulin clearance: (a) chromatogram of plasma before inulin injection; (b) $t=15$ min, 0.368 mg/ml; (c) $t=30$ min, 0.221 mg/ml; (d) $t=60$ min, 0.113 mg/ml; (e) $t=120$ min, 0.051 mg/ml; (f) $t=180$ min, 0.030 mg/ml; chromatograms at different times after inulin injection.

column composed by a cation- and an anion-exchange cartridge allows an effective deionization on-line of the sample resulting in a stable base line [21].

A positive relationship between temperature of the drift tube and response of ELSD up to 130°C maintaining the flow-rate of 0.6 ml/min and the carrier gas (air) of 3.5 slpm was found. Higher temperature decreases the detector response and increases the baseline noise.

In order to verify interference due to glucose and fructose, an aqueous solution containing 0.3 mg/ml each of inulin, glucose (retention time 12.5 min), fructose (retention time 17 min), and melezitose was analyzed. No interference with the recorded peaks was observed in the chromatogram. Since inulin is

used in the evaluation of peritoneal transport in patients on peritoneal dialysis, we tested a four-fold diluted dialysis solution containing 3.86 g/l of glucose before and after addition of 0.6 mg/ml of inulin. Even in this condition, the inulin peak is free of any interference.

Since mild acid conditions may hydrolyze inulin, the effects of the precipitating solution were also investigated. Inulin hydrolysis was excluded by the absence of fructose and glucose peaks in the chromatogram of an aqueous standard solution of inulin injected six h after deproteinization.

3.2. Calibration curves

The response of the ELSD detector is non-linear as a result of basic light-scattering principles. Calibration curves were plotted using double logarithmic co-ordinates according to the equation below [22]:

$$y = a \cdot xb \quad (1)$$

$$\log(y) = \log(a) + \log(x) \cdot b \quad (2)$$

where y is the detector response, x the sample concentration, and a and b are numerical coefficients. Calibration curves were plotted considering concentrations ranging from 0.05 to 1.2 mg/ml (0.05, 0.15, 0.3, 0.6, 1.2 mg/ml) both in plasma and urine. Each point was established from an average of five determinations.

Equation curves were $\log(y) = -1.8524 + 1.4431 \cdot \log(x)$ and $\log(y) = -1.7468 + 1.4683 \cdot \log(x)$ for plasma and urine, respectively. The correlation coefficient (r) was 0.999. The detection limit (signal-to-noise ratio = 3) was 5 μg/ml both in plasma and urine.

3.3. Precision

The within-day and the total (between-day) imprecision of the method were assessed by analyzing three plasma and urine samples with different inulin content ten times per day for 10 days (Table 1).

Table 1
Precision of the method in plasma and urine

Plasma ($n=10$)		Urine ($n=10$)	
Mean±SD (mg/ml)	C.V. (%)	Mean±SD (mg/ml)	C.V. (%)
Within-run			
0.052±0.001	3.4	0.052±0.002	3.5
0.149±0.004	3.2	0.297±0.006	2.1
0.301±0.008	2.6	1.204±0.020	1.8
1.201±0.018	1.7	2.399±0.029	1.5
Between-run			
0.053±0.003	4.3	0.052±0.003	4.4
0.151±0.007	4.5	0.298±0.010	3.5
0.299±0.013	3.9	1.201±0.025	2.3
1.200±0.024	2.0	2.397±0.046	2.0

3.4. Accuracy

Recovery was determined by addition of both plasma and urine samples with three known different amounts of inulin. The percentage of recovery was obtained by measuring inulin concentration in the samples before and after the addition. Analytical recovery ranged from 97.9 to 100.1% in plasma and from 99.1 to 99.7% in urine, respectively (Table 2).

3.5. Interference

Glucose, fructose and the co-administered drugs reported in Table 3, usually taken by patients with renal diseases, do not interfere with the assay.

3.6. Inulin clearance

GFR determined by inulin clearance and creatinine clearance is reported in Table 4.

4. Discussion

CIn is considered to be the ‘gold standard’ for both clinical and investigative assessment of GFR. Despite this, several factors such as hydration of patients, dose of inulin, posture, time delay between filtration at the glomerulus site and the bladder, number of blood samples, urine collection by spontaneous voiding, pharmacokinetic model are all well known to influence the clearance. Moreover, the reliability of both colorimetric and enzymatic methods of analysis is often unsatisfactory resulting in significant bias and interference from endogenous compounds [1,23–25].

Table 2
Analytical recovery of inulin in plasma and urine

Plasma ($n=10$)			Urine ($n=10$)		
Added (mg/l)	Found (Mean±SD)	Recovery (%)	Added (mg/l)	Found (Mean±SD)	Recovery (%)
0.150	0.143±0.010	97.9	0.300	0.294±0.016	99.2
0.300	0.296±0.011	99.2	0.600	0.591±0.024	99.1
1.200	1.202±0.024	100.1	1.200	1.195±0.030	99.7

In an attempt to improve analytical reliability, several HPLC procedures have been published in the last years. All those HPLC methods show a better sensitivity and precision and the advantage of eliminating interference due to plasma hexoses. However, one procedure needs the acid hydrolysis of inulin to fructose and its conversion to hydroxymethylfuraldehyde [17] while the other requires a long extraction procedure before the HPLC analysis of the sample [18,19].

We present here an HPLC method that has the advantage of being rapid, precise and accurate and to measure inulin without previous hydrolysis and/or cartridge extraction of the sample. The use of phosphotungstic acid and zinc acetate [21] represents an excellent alternative of solid-phase extraction since it allows a fast and economic deproteinization of the samples.

The choice of the reagent volume for sample pre-treatment represents the critical step of the

Table 3
Compounds tested for interference

Compound	Concentration (mg/l)
Ampicillin	250
Azathioprine	40
Atenolol	1000
Calcitriol	4
Calcium Carbonate	1000
Cephazolin	2500
Cephtazidim	1000
Cyclosporine	2000
Diazepam	100
Dipyridamol	75
Erytropoietin (×U/ml)	*2000
Gentamycin	500
Labetalol	1000
Nifedipine	100
p-aminobenzoic acid	1000
p-aminohippuric acid	1000
Ranitidine	1100
Teophilline	120

Table 4
Glomerular filtration rate evaluated with inulin clearance and creatinine clearance (method of Schwartz)

Inulin clearance (ml/min/1.73 m^2)	Creatinine clearance (ml/min/1.73 m^2)
104	100
84	100
91	102
97	134
105	103
103	109
83	88
103	96
99	96
104	109
85	117
115	96
112	104
103	89
135	81
130	95
80	95
117	115
77	90
96	116
94	102
83	88
113	89
124	114
83	117
95	100
78	96
145	95
76	89
139	103
94	88

analysis. Volumes which are too low cause inappropriate sample purification and a rapid reduction of column performances; while volumes that are too high induce a deterioration of the deashing system after a few injections.

The volume of 30 μl of precipitating reagent represents a compromise between the need of obtaining a good inulin peak separation and to preserve the column performances. The use of the Biorad's in-line carbohydrate deashing column, equipped with an anion-exchange Micro-Guard cartridge, is necessary to obtain an optimal nebulization of ELSD. The pre-column system allows performance of up to 200 injections without appreciable baseline drift or interference with inulin and I.S. peaks.

In order to prevent variations of retention time and shape of the recorded peaks in the chromatograms, the analytical column requires a periodical (every 200 injections) regeneration procedure with lead nitrates 0.1 mol/l.

The sample preparation step comprising deproteinization and filtration is very simple, and a series of 24 samples can be prepared simultaneously.

Even though the analytical recovery of inulin is satisfactory the use of melezitose as internal standard reduces the within-day and the total (between-day) imprecision. Drugs usually administered to patients with renal failure, at therapeutic levels, did not interfere with the assay.

The method is very sensitive and only 100 μl plasma are sufficient for the assay. The small volumes of sample required are also very important when GFR has to be monitored in children and in experimental models. Additionally, the cost of the procedure is low and the total analytical time very short, particularly when an autosampler is available.

In conclusion, the described method could represent a useful tool for measuring inulin in biological fluids, particularly in children and for experimental purposes.

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