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Short communication

Simultaneous determination of inulin and *p*-aminohippuric acid in plasma and urine by reversed-phase high-performance liquid chromatography

Anna Pastore[a], Sergio Bernardini[a,c], Luca Dello Strologo[b], Gianfranco Rizzoni[b], Claudio Cortese[c], Giorgio Federici[a,c,*]

[a]*Laboratory of Clinical Biochemistry, Children's Hospital and Research Institute "Bambino Gesù", Piazza S. Onofrio 4, 00165 Rome, Italy*

[b]*Department of Nephrology and Dialysis, Children's Hospital and Research Institute "Bambino Gesù", Piazza S. Onofrio 4, 00165 Rome, Italy*

[c]*Department of Internal Medicine, University of Rome "Tor Vergata", Via di Tor Vergata 135, 00133 Rome, Italy*

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Abstract

A simple, accurate and sensitive high-performance liquid chromatographic method with UV detection was carried out to measure simultaneously plasma and urine concentrations of both *p*-aminohippuric acid and inulin. Following a simplified acid hydrolysis of the sample, the separation was carried out in 4 min using a C_{18} reversed-phase column with a flow-rate of 1 ml/min, and monitoring the absorbance at 280 nm. Within the investigated concentration ranges of inulin (0.1–3.2 mg/ml) and *p*-aminohippuric acid (0.0097–0.3 mg/ml), good linearity ($r>0.99$) was obtained. Within-run RSD ranged from 2.9 to 6.1% and between-run RSD ranged from 6.4 to 10%. Analytical recoveries were 101–112%, with little differences between plasma and urine samples. The detection limit was 1 μg/ml for all the analytes studied. This method might be ideal for renal function studies where a rapid and reproducible assessment of both renal glomerular filtration rate and blood flow-rate is required. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Inulin; *p*-Aminohippuric acid

1. Introduction

The assessment of glomerular filtration rate (GFR) is an important parameter used to evaluate the renal function and its response to treatments in patients with kidney diseases. Inulin clearance (C_{in}) is considered 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 [1,2].

p-Aminohippuric acid [PAH, *N*-(4-aminobenzoyl)-glycine] is used to determine the effective

*Corresponding author. Laboratory of Clinical Biochemistry, Children's Hospital and Research Institute "Bambino Gesù", Piazza S. Onofrio 4, 00165, Rome, Italy. Tel.: +39-6-6859-2210.

E-mail addresses: giorgio.federici@uniroma2.it (G. Federici), federici@obg-irccs.rm.it (G. Federici).

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renal plasma flow (ERPF) being freely filtered at the glomerular level but it is also extensively secreted and very poorly reabsorbed within the tubules. Several high-performance liquid chromatography (HPLC) methods had been developed to measure the concentration of inulin or *p*-aminohippuric acid in biological fluids [3–8], but no methods report the simultaneous assay for inulin and PAH using reversed-phase HPLC.

We described here a rapid and sensitive HPLC method for the simultaneous measurement of inulin and *p*-aminohippuric acid in plasma and urine after a simplified acid hydrolysis of the sample.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade and were readily available commercial products.

2.2. Equipment

The HPLC system, with an autosampler and a solvent delivery system, was a Hewlett-Packard Model 1090 M Aminoquant Series II; the diode-array detector was a Hewlett-Packard Model 79880 operating at a wavelength of 280 nm; the data obtained were analyzed with the HP Chemstation program (Hewlett-Packard, Amsterdam, The Netherlands).

2.3. Sample preparation and HPLC analysis

Samples were prepared as described by Dall'Amico et al. [5] with minor modifications; 100 μl of undiluted plasma or standards or 1:10 (v/v) diluted urine with saline solution were spiked with 100 μl of the working internal standard, *p*-aminobenzoic acid (PABA, 5 μg/ml in distilled deionized water) and 100 μl of $HClO_4$/water (70:30, v/v). The solutions were vortex-mixed for 10 s and centrifuged at 1000 *g* for 5 min; the supernatant was boiled for 10 min to hydrolyze inulin to fructose and to convert fructose to hydroxymethylfuraldehyde (HMF), and to remove the acetyl residue from the acetylated moiety of *p*-aminohippuric acid [5,9]. The samples were cooled on ice for 5 min before analysis.

A 10-μl volume of sample was injected into a Nova-Pak C_{18} column (150×3.9 mm, 4 μm particle size; Waters, Milford, MA, USA) equilibrated with 3.2 mmol/l HCl, pH 2.5 (A). The compounds were eluted from the column in 4 min at ambient temperature with a gradient of 3.2 mmol/l HCl–acetonitrile (40:60, v/v), pH 2.5 (B) (0 min, 5% B; 1–4 min, 15%) at a flow-rate of 1 ml/min. The column equilibration time was 2 min.

3. Results and discussion

Fig. 1A shows the chromatogram of the components of a standard solution. Under the conditions used, eluted peaks were distinctly separated. Unidentified peaks appeared which did not interfere with the peaks of interest. The retention times of *p*-aminohippuric acid and inulin were 2.4 and 2.8 min, respectively. Fig. 1B and C shows chromatograms of plasma sample and urine sample, respectively, of a subject after intravenous (i.v.) somministration of known concentrations of inulin and *p*-aminohippuric acid.

Table 1 reports the results of precision of the assay. The intra-assay precision was obtained by analyzing 10 replicates of the biological samples on the same day. The inter-assay precision was determined by analyzing the same biological samples on 10 different days over 1 month. Known concentrations of inulin and *p*-aminohippuric acid were added to plasma or urine sample for recovery studies. The concentrations in biological samples with added standards were determined in five replicates and analytical recoveries were calculated (Table 2). Calibration curves for each analyte (0.1–3.2 mg/ml inulin, $n=8$; 0.0097–0.3 mg/ml *p*-aminohippuric acid, $n=8$) were prepared in duplicate by diluting the stock solutions with water. The linearity of the assays was also studied in the following ranges: 0–10 mg/ml inulin and 0–1 mg/ml PAH, prepared diluting stock solutions in pooled plasma or diluted urine. A linear relation was obtained between peak area ratios of inulin and PAH to PABA and the expected compounds concentration. Correlation coefficients were >0.99 for all analytes.

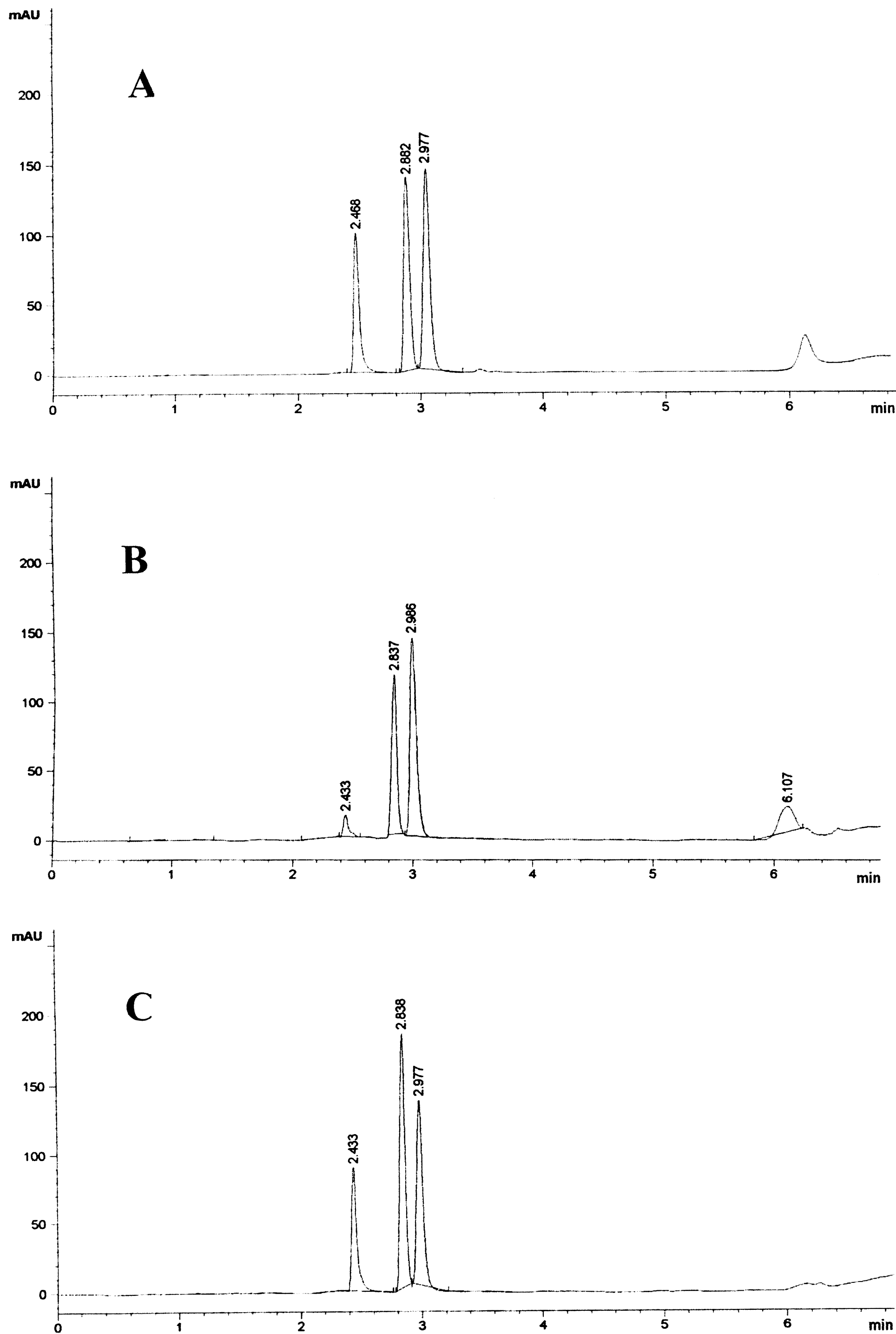

Fig. 1. Chromatograms of (A) standard solution containing 0.0625 mg/ml of PAH and 0.125 mg/ml of inulin; (B) human plasma of a subject after intravenous somministration of known concentrations of inulin and *p*-aminohippuric acid, spiked with 0.005 mg/ml of internal standard; (C) human urine of a subject after intravenous somministration of known concentrations of inulin and *p*-aminohippuric acid, spiked with 0.005 mg/ml of internal standard. Retention times were 2.4 min for PAH, 2.8 min for inulin and 2.9 min for internal standard (PABA).

Table 1
Precision of the assay in plasma and urine

	Intra-assay (n=10)			Inter-assay (n=10)		
	Mean (μg/ml)	SD	RSD (%)	Mean (μg/ml)	SD	RSD (%)
Plasma						
p-Aminohippuric acid	0.034	0.001	2.9	0.031	0.002	6.4
Inulin	0.3	0.01	3.3	0.028	0.02	7.1
Urine						
p-Aminohippuric acid	0.053	0.002	3.7	0.05	0.004	8.0
Inulin	0.13	0.008	6.1	0.15	0.015	10.0

The equations for the regression line (n=8) were: y=0.13x+0.05 for inulin; y=0.37x+0.08 for PAH in urine, and y=0.96x+0.019 for inulin and y=0.96x+0.08 for PAH in plasma (where y is the peak area and x is the concentration of the analyte). The limit of detection for standard samples, defined as the concentration that produces a signal-to-noise ratio >5, was about 1 μg/ml for PAH and 50 μg/ml for inulin, both in plasma and urine.

For scientific reasons, precise evaluation of GFR is always necessary. In clinical practice this technique is only used in selected cases: a possible example is reported below.

A 18-year-old boy with chronic renal failure following focal and segmental sclerosis had a creatinine clearance persistently between 15 and 20 ml/min/1.73 m^2. This degree of renal failure is generally managed with conservative treatment, but this boy required chronic dialysis due to hyperkalemia and fluid retention. To measure exact renal function inulin and PAH clearances were performed.

Dosages were calculated in order to maintain PAH plasma levels below 50 mg/l and inulin below 250 mg/l. A 1-h equilibration period was allowed for infusion stabilization before starting the measurement. The test lasted for 2 h. Plasma was collected at the beginning and the end of each clearance period with the mean of PAH and inulin levels used for calculation of clearances.

Clearances were obtained by simultaneous plasma

Table 2
Recovery of the assay

	Sample (mg/ml)	Added (mg/ml)	Measured[a] (mg/ml)	Mean recovered (%)
Plasma				
p-Aminohippuric acid	0.033	0.005	0.04 (0.001)	105
		0.5	0.54 (0.02)	101
		5	5.10 (0.25)	101
Inulin	0.45	0.05	0.48 (0.02)	96
		0.5	1.01 (0.06)	105
		5	5.6 (0.18)	112
Urine				
p-Aminohippuric acid	0.06	0.005	0.062 (0.002)	95
		0.5	0.535 (0.03)	106
		5	5.3 (0.2)	105
Inulin	0.15	0.05	0.22 (0.01)	110
		0.5	0.7 (0.04)	108
		5	5.4 (0.2)	105

[a] Mean of five replicate values; SD are indicated in parentheses.

and urine collection during a continuous infusion of both inulin and PAH.

The resulting GFR was 8 ml/min/1.73 m^2 and effective renal plasma flow was 25 ml/min/m^2. This confirmed the clinical need for dialysis. The discrepancy between inulin and creatinine clearance was attributed to a high creatinine tubular secretion which increased the clearance. It must be considered that tubular secretion of creatinine increases proportionally with declining levels of renal function [10].

In conclusion, this method provides a simultaneous reliable determination of both inulin and PAH in plasma and urine allowing thus a precise determination of GFR and ERPF for any degree of renal failure.

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