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Simultaneous determination of inulin and *p*-aminohippuric acid (PAH) in human plasma and urine by high-performance liquid chromatography

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Inulin and *p*-aminohippuric acid (PAH) clearances are used for the estimation of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF). A simple and rapid high-performance liquid chromatography (HPLC) method with UV detection is described for the simultaneous determination of inulin and PAH in the same chromatogram in the plasma and urine of humans. Plasma and urine samples were hydrolyzed with perchloric acid (0.7%) in boiling water. The mobile phase consisted of 0.01 M potassium dihydrogenphosphate with 0.02 M tetramethylammonium chloride and *o*-phosphoric acid (pH 3)–acetonitrile (94 : 6, v/v), pumped at a rate of 1.2 ml min^{-1} on a C_8 reversed-phase column. Tannic acid was used as the internal standard and UV detection at 285 nm was employed. The calibration curves were linear over the concentration range of 12.5–100 mg l^{-1} for inulin and 6.25–50 mg l^{-1} for PAH with determination coefficients greater than 0.997. The method is accurate (bias $< 13\%$) and reproducible (intra- and inter-day relative standard deviation less than 11%), with a limit of quantitation of 12.5 mg l^{-1} and 6.25 mg l^{-1} for inulin and PAH, respectively. Analytical recoveries from urine and plasma were ranged from 81 to 108% for both compounds. This fully validated method, which allows the simultaneous determination of inulin and PAH clearances, is simple, rapid (total run time < 10 min) and requires only a 200 μl plasma or urine sample.

Introduction

Determination of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) is essential for the study of renal hemodynamics. Serum creatinine and creatinine clearance are poor markers of GFR and can be influenced by a variety of drugs and glomerular diseases.[1,2] Traditionally, inulin and *p*-aminohippuric acid (PAH) clearances are used for the measurement of GFR[3] and ERPF,[4,5] respectively.

However, the determination of these two markers has been a problem for a long time because of the lack of satisfactory analytical methods. Inulin being a polysaccharide, which on hydrolysis yields fructose, most inulin colorimetric tests are based on reactions with the fructose derived from acid hydrolysis of inulin.[6] These methods appear time-consuming and often unreliable, mainly due to interference with other plasmatic hexoses.[7] The measurement of PAH by colorimetric reaction is often difficult and frequently inaccurate in the presence of glucose and certain drugs.[8–10] In recent years, several high-performance liquid chromatographic (HPLC) techniques have been developed for inulin and PAH quantitation but are not without problems.[10] Indeed, those methods usually require extensive extraction procedures,[9,11] utilize large sample volumes,[9] require the use of dual-wavelength ultraviolet detection[12] or a limited injection volume because of peak splitting.[13] Recently, interesting sensitive and specific HPLC procedures have been reported[6,10,14] but, to date, none of them has the capability of measuring inulin and PAH simultaneously in biological matrices.

This paper describes a simple and fast reversed-phase HPLC method that allows the simultaneous measurement of inulin and PAH in plasma and urine of humans after a single-step acid hydrolysis of the sample.

Experimental

Reagents

4-Aminohippuric acid (PAH) and tannic acid [the internal standard (IS)] were purchased from Sigma (St. Louis, MO, USA) and inulin from Serb (Paris, France). Perchloric acid ($HClO_4$), tetramethylammonium chloride and potassium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile was purchased from SDS (Peypin, France) and *o*-phosphoric acid purchased from Prolabo (Paris, France). Water was obtained from a Milli-Q Waters purification system (Millipore, Saint-Quentin-Yvelines, France).

Apparatus and chromatographic conditions

The liquid chromatograph consisted of a Varian Model 9012 HPLC pump and a manual Rheodyne 7125 injection valve equipped with a 20 μl loop. The chromatographic separations were carried out using a 5 μm particle size Lichrospher column 100 RP8 (250 × 4 mm id, Merck, Nogent sur Marne, France). The separated components were detected using a Spectroflow 783 UV detector (Eurosep Instruments, Cergy-Pontoise, France) and detector signals were recorded on a Kipp and Zonen chart-recorder (Touzart et Matignon, Courtaboeuf, France). The mobile phase consisted of 6% acetonitrile in 0.01 M potassium dihydrogenphosphate containing 0.02 M tetramethylammonium chloride and adjusted to pH 3.0 with *o*-phosphoric acid. The flow-rate was 1.2 ml min^{-1} and the wavelength set at 285 nm. All analyses were performed at room temperature.

Solutions' preparation

Stock solutions of inulin were prepared as 200 mg l^{-1} in purified water by immersing the flask in boiling water to obtain total dissolution. Stock solutions of PAH (100 mg l^{-1}) and tannic acid (IS) (1 g l^{-1}) were also made in purified water. The solutions were stored at +4 °C. Standard solutions were freshly prepared for each run day.

Standards and quality control

A working solution was prepared by mixing the stock solutions of inulin and PAH (50:50, v/v). Working standards were prepared from this solution by serial dilution with purified water to yield concentrations of 12.5, 25, 50 and 100 mg l^{-1} for inulin and 6.25, 12.5, 25 and 50 mg l^{-1} for PAH both for plasma and urine determinations.

Inulin and PAH quality controls have been prepared with a plasmatic pool of patients. Plasma aliquots were stored at −20 °C until assay.

Sample preparation

Aliquots (200 μl) of standards, subject plasma and urine samples (respectively diluted 1:2 and 1:50 with purified water) were transferred into clean conical tubes (SGE France Sarl, Villeneuve-Saint-Georges, France). To each were added 50 μl of the IS stock solution and 100 μl of perchloric acid (0.7%). The tubes were vortexed briefly, left in boiling water for 60 min to hydrolyze inulin to fructose and to convert fructose to 5-(hydroxymethyl)-2-furaldehyde (HMF). Then, the samples were cooled in cold water for 5 min. After centrifugation (4000*g*, 5 min), 20 μl of the clean supernatant were injected into the HPLC system.

Calculations

Quantitative analyses of inulin and PAH were performed using the internal standard method. Standard curves were obtained by unweighted linear regression of the peak-height ratios of HMF and PAH to IS *versus* known concentrations of inulin and PAH. Concentrations of quality controls and unknown samples were estimated by applying the linear regression equation of the standard curve to the unknown samples' peak-height ratios.

Recovery of PAH and inulin

Values of percentage recoveries were determined by comparing the peak heights of urine or plasma blank samples spiked with different amounts of inulin or PAH and treated as any sample, with the peak heights of the same standards prepared in purified water. Each point was established from an average of 6 determinations.

Precision and accuracy

Precision and accuracy of the assay were assessed by replicate analyses of spiked urine or plasma blank samples over the concentration range of inulin and PAH used for calibration curves. Seven and six separate samples at each concentration level were assayed for inter-day and intra-day evaluations, respectively. Precision is reported as percentage relative standard deviation (RSD) of the estimated concentrations and accuracy (bias) expressed as [(mean calculated concentration/spiked concentration) × 100 − 100]. Precision was also assessed for the plasmatic pool analyzed with each analytical run.

Results and discussion

Chromatography

Representative chromatograms of patient plasma (Fig. 1b) and urine (Fig. 1c), prior to and after receiving an intravenous injection of inulin and PAH, are compared with a chromatogram of an aqueous standard solution (Fig. 1a) of inulin (100 mg l^{-1}), PAH (50 mg l^{-1}) and IS (250 mg l^{-1}). The peaks of PAH and inulin as HMF are clearly resolved with short retention times of 4.30 and 9 min, respectively. Intermediate retention time (5.30 min) is obtained for the IS (tannic acid).

The chromatographic profiles of both plasma and urine samples do not show any interfering substance at the retention time corresponding to the recorded peaks. However, a small unknown peak may occasionally (≈1/100 analyses) interfere at the HMF retention time. Changes in analytical conditions were made in an attempt to minimize this interference while maintaining the simultaneous determination of PAH and HMF. As this was not successful, the residual unspecific value, when present in blank plasma or urine, was subtracted from all subsequent analyses.

The mobile phase was chosen to provide concomitantly the best peak resolution and retention times. The pH of the mobile phase was found to play an important role in peak resolution. It was first fixed at 4.8 but then lowered to 3.0 so that the peaks were not too close. Finally, the UV absorbance was set at 285 nm as a compromise to obtain reasonable peak heights for the three compounds. In fact, the use of 240 or 254 nm wavelengths was responsible for excessively small peak heights which caused us to increase the sensitivity.

The use of automatic injection of the samples, together with the lack of extraction, allows an operator to analyze 48 plasma and/or urine samples within an 8 h working day.

Linearity

The linearity of the method was evaluated with calibration curves made in purified water ranging from 6.25 to 50 mg l^{-1}

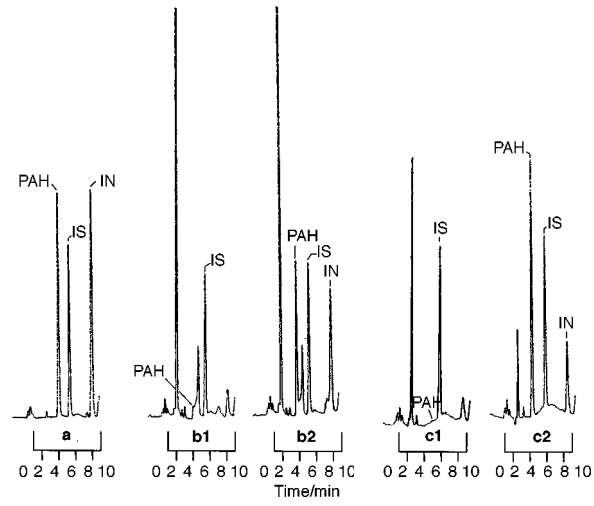

Fig. 1 Representative chromatograms of an aqueous standard solution (panel a) of inulin as HMF (IN) (100 mg l^{-1}), PAH (50 mg l^{-1}) and tannic acid (IS) (250 mg l^{-1}) compared with diluted plasma (panel b, dilution factor 1:2) and urine (panel c, dilution factor 1:50) collected before (panels b1 and c1) and after (panels b2 and c2) an intravenous injection of PAH and inulin in one volunteer.

for PAH and from 12.5 to 100 mg l^{-1} for inulin. Each point was established from an average of 16 determinations.

For both compounds, a linear relationship between detector signal and spiked concentrations was found, as described by the following linear regression equations: $y = 0.026x + 0.013$ ($r^2 = 0.997$) for PAH and $y = 0.020x - 0.004$ ($r^2 = 0.998$) for inulin, where y is the inulin or PAH concentration (mg l^{-1}) and x is the peak-height ratio of HMF or PAH to IS. Values of the coefficients of determination are satisfactory and none of the intercepts was significantly different from zero. The latter indicates the lack of interfering peaks when calibration curves are assessed in purified water. The limit of detection was 0.5 mg l^{-1} (signal-to-noise: 3) for HMF and PAH in both plasma and urine. The limit of quantitation (LOQ) for inulin and PAH in plasma and urine was chosen as the concentration used for the lowest concentration level on the calibration curves. The LOQ seems to be adequate taking into account the concentrations of inulin and PAH determined in samples collected in a preliminary clinical investigation in subjects conducted in our laboratory (data not shown).

Analytical recovery

The percentage recoveries (mean value ± s, $n = 6$) of inulin and PAH obtained in spiked plasma and urine over the whole concentration range are given in Table 1.

Intra-day and inter-day precision and accuracy

Within-assay and between-assay precisions and accuracies for all the range of concentrations of inulin and PAH in both matrices are reported in Tables 2 and 3. The results demonstrate that the precision and accuracy of the method are good over the range of concentrations studied.

The quality control constituted by a plasmatic pool of patients has been assessed over 38 analyses. The results (Table 4) show that the RSD values remain satisfactory.

Comparison of colorimetric and HPLC methods for the determination of PAH and inulin in biological fluids

Colorimetric assays are commonly used for the determination of inulin and PAH in biological fluids. Classical inulin colorimetric methods entail initial acidic or enzymatic hydrolysis of inulin to fructose.[11] It is then made to react with a substrate to produce a colored reaction compound that can be measured photometrically. Despite differences in the substrates used, these methods usually overestimate inulin concentrations because other compounds, such as glucose, may react with the chromogenic reagent.[6,11] In the same way, PAH colorimetric assay is also limited by the lack of specificity in the presence of glucose or certain drugs, such as sulfonamides, or some local anesthetics.[10,14] In addition, some drugs administered to patients with renal failure may interfere with the inulin or PAH assay.[6] Although, several techniques have been used to reduce these interferences, all of those additional steps require labor

Table 1 Analytical recovery of inulin and PAH in plasma and urine ($n = 6$)

PAH			Inulin		
Spiked plasma concentration/mg l^{-1}	Recovery (%)	RSD[a] (%)	Spiked plasma concentration/mg l^{-1}	Recovery (%)	RSD[a] (%)
6.25	92.4 ± 4.2	4.5	12.5	102.3 ± 5.6	5.5
12.5	96.5 ± 3.4	3.6	25	81.3 ± 3.0	3.8
25	98.9 ± 4.0	4.1	50	102.9 ± 2.0	2.0
50	108.0 ± 4.9	4.6	100	103.7 ± 7.2	6.9
Spiked urine concentration/mg l^{-1}	Recovery (%)	RSD[a] (%)	Spiked urine concentration/mg l^{-1}	Recovery (%)	RSD[a] (%)
6.25	89.7 ± 1.5	1.7	12.5	85.0 ± 4.6	5.4
12.5	91.0 ± 2.5	2.7	25	91.0 ± 2.7	3.0
25	91.6 ± 1.2	1.3	50	92.3 ± 2.6	2.8
50	93.1 ± 0.9	1.0	100	93.1 ± 0.9	1.0

[a] Relative standard deviation.

Table 2 Intra-day and inter-day precision and accuracy of PAH and inulin in plasma

PAH				Inulin			
Inter-day ($n = 7$)	Mean ± s/mg l^{-1}	RSD[a] (%)	Bias[b] (%)	Inter-day ($n = 7$)	Mean ± s/mg l^{-1}	RSD[a] (%)	Bias[b] (%)
6.25	6.27 ± 0.57	9.1	0.3	12.5	13.53 ± 1.47	10.9	8.2
12.5	13.37 ± 0.29	2.2	6.9	25	26.23 ± 2.22	8.5	4.9
25	27.83 ± 2.23	8.0	11.3	50	53.49 ± 1.70	3.2	6.9
50	56.41 ± 3.69	6.5	12.8	100	102.97 ± 6.35	6.1	2.9
Intra-day ($n = 6$)				Intra-day ($n = 6$)			
6.25	6.88 ± 0.33	4.8	10.1	12.5	13.85 ± 1.06	7.6	10.8
12.5	13.10 ± 0.92	7.0	4.8	25	29.01 ± 1.08	3.7	16.0
25	25.20 ± 0.41	1.6	0.8	50	53.44 ± 3.12	5.8	6.9
50	48.19 ± 2.04	4.2	−3.6	100	104.45 ± 4.04	3.8	4.4

[a] Relative standard deviation. [b] Expressed as [(mean calculated concentration/spiked concentration) × 100 − 100].

Table 3 Intra-day and inter-day precision and accuracy of PAH and inulin in urine

PAH					Inulin				
Inter-day	*n*	Mean ± *s*/mg l^{-1}	RSD[a] (%)	Bias[b] (%)	Inter-day	*n*	Mean ± *s*/mg l^{-1}	RSD[a] (%)	Bias[b] (%)
6.25	25	6.12 ± 0.41	6.7	−2.1	12.5	25	13.08 ± 0.99	7.6	4.6
12.5	26	12.55 ± 0.51	4.1	0.4	25	26	25.28 ± 1.43	5.7	1.1
25	26	25.18 ± 1.09	4.3	0.7	50	25	49.28 ± 1.63	3.3	−1.4
50	26	49.97 ± 0.49	1.0	−0.1	100	25	100.16 ± 0.90	0.9	0.2
Intra-day					Intra-day				
6.25	6	5.99 ± 0.31	5.2	−4.2	12.5	6	13.24 ± 0.48	3.6	5.9
12.5	6	12.77 ± 0.76	5.9	2.2	25	6	24.98 ± 1.12	4.5	−0.1
25	6	25.52 ± 1.07	4.2	2.1	50	6	48.33 ± 3.49	7.2	−3.3
50	6	49.62 ± 2.64	5.3	−0.8	100	6	100.75 ± 3.32	3.3	0.8

[a] Relative standard deviation. [b] Expressed as [(mean calculated concentration/spiked concentration) × 100 − 100].

Table 4 Precision of the plasmatic quality control

	n	Mean ± *s*/mg l^{-1}	RSD[a] (%)
PAH	38	44.8 ± 5.8	15.7
Inulin	37	112.0 ± 17.6	12.9

[a] Relative standard deviation; *n* number of different experiments.

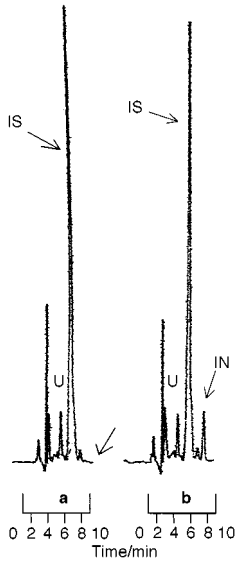

Fig. 2 Chromatograms of diluted human blank plasma spiked with IS before (panel a) and after addition of inulin (IN) (12.5 mg l^{-1}) (panel b). Samples were treated in accordance with the procedure described under Sample preparation (100 µl of 0.7% perchloric acid, 60 min in boiling water). The peak corresponding to inulin (IN) is HMF. Glucose does not interfere at the HMF retention time (indicated by arrows at retention time of 9 min). U indicates an unidentified peak found in plasma.

intensive sample preparation and may sometimes be the source of additional errors in the procedure.[6]

It is well known that hexoses heated in acid solution yield hydroxymethylfuraldehyde. An analogous reaction is carried out in the method described in this paper, since it is based on the HPLC measurement of HMF formed by the acid hydrolysis of inulin at high temperature. As described in a previously published method,[6] we initially used concentrated acid (70% $HClO_4$) to perform inulin hydrolysis. Since glucose was also converted into HMF, and thus may have produced marked interference with the HMF peak in our assay, the conditions were modified by using diluted $HClO_4$ (0.7%, v/v, in water). In later experiments, glucose was not transformed to HMF, and the background interference likely to distort measurement accuracy for samples containing low inulin concentrations disappeared as shown in plasma in Fig. 2.

Although numerous HPLC methods for inulin and PAH estimation have been published over recent years, none of them has reported the simultaneous determination of inulin and PAH in biological fluids. Therefore, this is the first reported HPLC method enabling the concomitant quantitation of inulin and PAH in the same chromatogram in the plasma and urine of humans. This technique is simple, requires only 200 µl of sample, and has a short analysis duration, particularly when an autosampler is available.

In conclusion, this accurate, precise and fast method could represent a useful tool for the assessment of inulin and PAH clearances in humans.

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