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High-performance liquid chromatographic determination of *p*-aminohippuric acid and iothalamate in human serum and urine: comparison of two sample preparation methods

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Abstract

A high-performance liquid chromatography method applied to determine *p*-aminohippuric acid (PAH) and iothalamate (IOT) in serum and urine samples of patients was evaluated according to recovery, reproducibility and linearity utilizing narrow-bore columns. The mobile phase consisted of 0.15 *M* sodium dihydrogenphosphate with 1.2 m*M* tetrabutylammonium sulphate, the pH was adjusted to pH 4.6, acetonitrile was added to a final ratio of 95:5 (v/v), the flow-rate was set at 0.3 ml/min. The separation was achieved on a ODS Hypersil column (200×2.1 mm I.D.). The UV detector was set at 254 nm. PAH and IOT are used for evaluation of kidney function [effective renal plasma flow (ERPF) and glomerular filtration rate (GFR)]). Under the described chromatographic conditions two sample preparation techniques, ultrafiltration and acetonitrile precipitation were compared. The results demonstrate the accuracy of both methods in evaluation of ERPF and GFR. Due to its cost-effectiveness we recommend the acetonitrile precipitation method in clinical routine.

Keywords: *p*-Aminohippuric acid; Iothalamate

1. Introduction

The accurate determination of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) is essential to evaluate kidney function. Non-invasive and non-isotopic methods are preferred to avoid unjustified risks and complex diagnostic procedures. Unfortunately no endogenous substances have been deemed suitable for the evaluation of renal clearance [1]. Therefore exogenous compounds such as inulin for determination of GFR and *p*-aminohippuric acid (PAH) for evaluation of ERPF have been used as markers. Due to laborious sample preparations, the non-specificity of the colorimetric assay, and the need for a feasible diagnostic system for daily clinical routine, inulin was substituted by radioactive-labelled iothalamate (IOT) [2,3]. In combination with radiolabelled *p*-aminohippuric acid, radiola-

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belled IOT is used for measuring GFR and ERPF [4–6]. However radiation exposure and disposal of radioactive waste are undesirable. Furthermore prolonged elimination of the radiolabelled substances and radioactive decay limit repetitive investigations within a short period. Therefore several reversed-phase high-performance liquid chromatography (HPLC) methods were developed to simultaneously measure non-radioactive iothalamate and *p*-aminohippuric acid in plasma or serum and urine utilizing *p*-aminobenzoic acid (PABA) as an internal standard [4–8].

Initial methods for sample preparation consisted of several time-consuming extraction and evaporation steps. Later developed procedures reduced sample preparation to a single precipitation step or an ultrafiltration step prior to analysis [5,9]. Herein we report on a method using narrow-bore columns, which allow estimation of PAH and IOT in a single chromatographic run, as well as on the accuracy of the two simple sample preparation methods, ultrafiltration vs. acetonitrile (ACN) precipitation.

These sample preparation methods are modifications of Bell et al. for the precipitation method [7], and of Lowe et al. [8], Morelli et al. [9] and Oberbauer et al. [10] for the ultrafiltration method. Our aim was to compare these two described sample preparation methods using the internal standard aminobenzoic acid for accuracy and reproducibility. The results should indicate whether the inexpensive ACN precipitation method allows an accurate determination of kidney function.

2. Experimental

2.1. Reagents

Iothalamate meglumine (CONRAY 60) was obtained from Mallinckrodt Medical (Hennef/Sieg, Germany), PAH was purchased from Merck (West Point, PA, USA), PABA was from Sigma (St. Louis, MO, USA), sodium dihydrogenphoshate (suprapure), tetrabutylammonium sulphate (LiChropur) and acetonitrile (LiChrosolv gradient grade) were obtained from Merck (Darmstadt, Germany). For preparation of solutions Milli-Q water (Millipore, Bedford, MA, USA) was used. The ultrafiltration tubes (Centricon-10 filtration tubes; cut-off molecular mass$<$10 000) were obtained from Amicon (Beverly, MA, USA).

2.2. Apparatus

The analyses were performed on 1050-Series HPLC modules from Hewlett-Packard (Waldbronn, Germany), consisting of a quaternary pump, an autosampler and a multi-wavelength UV detector. A narrow-bore ODS Hypersil column, 5 μm, 200×2.1 mm I.D. from Hewlett-Packard was used for separation. The pump was set at a flow-rate of 0.3 ml/min. The standard injection volume was 20 μl. The column effluent was analyzed at a wavelength of 254 nm.

2.3. Mobile phase

The mobile phase consisted of 0.15 *M* sodium dihydrogenphosphate with 1.2 m*M* tetrabutylammonium sulphate, the pH was adjusted to pH 4.6. Acetonitrile was added to yield a final buffer–acetonitrile concentration ratio of 95:5 (v/v).

2.4. Preparation of standard solution

Stock solutions in Milli-Q water were 10 mg/ml for PAH, 20 mg/ml for IOT and 2 mg/ml for PABA. Standards of PAH and IOT in serum and urine were prepared by diluting the stock solution with water and addition of 50 μl of the dilutions to normal-pooled serum and normal-pooled urine from healthy subjects, to yield the final serum and urine sample concentrations of PAH–IOT of 25:50, 12.5:25, 6.25:12.5 and 3.125:6.25 μg/ml, respectively.

The stock solution of the internal standard PABA was diluted with water to yield a concentration of 0.2 mg/ml. All standards were aliquoted and stored at −70°C.

2.5. Preparation of samples

The preparation of all serum and urine samples was performed in a strictly parallel fashion (acetonitrile precipitation vs. ultrafiltration). Urine samples were diluted 1:40 prior to parallel preparation.

Aliquots of a sample (250 μl) were spiked with 50 μl of the internal standard and vigorously vortexed.

Corresponding samples were split for parallel preparation.

(a) Acetonitrile was added 1:1 for precipitation, the sample was vortexed and centrifuged (Eppendorff centrifuge at 4°C and 9600 *g*). The supernatant (250 μl) was diluted with 750 μl of acetonitrile-free buffer and an aliquot was transferred into an autosampler vial and sealed.

(b) Prior to ultrafiltration 250 μl of the mobile phase was added to the sample (250 μl) to equalize the dilution factor of the precipitation method. The samples were vortexed and 250-μl aliquots were further diluted 1:4 with acetonitrile-free mobile phase prior to ultrafiltration (Centricon-ultrafiltration units). After vortexing the ultrafiltration units were centrifuged at 3500 *g* for 15 min at 4°C. The filtrate was placed in an autosampler vial and sealed.

2.6. Recovery and linearity

The linearities of all calibration curves were determined between 0.125 and 50 μg/ml for PAH and 0.250 to 100 μg/ml for IOT, respectively. As we used concentrations for drug infusion into patients according to previous protocols, the linear range of four samples for the higher concentration levels (3.125 to 25 μg/ml for PAH and 6.25 to 50 μg/ml for IOT) are shown in the Results section. From these data mean values, standard deviations (SDs) and relative standard deviation (RSDs) were calculated.

3. Results and discussion

The top panels (A) in Fig. 1 illustrate chromatograms of human serum and urine samples supplemented with PAH, IOT and PABA (standard chromatograms) after acetonitrile precipitation. The middle panel (B) shows adequate samples taken from patients using the same sample preparation procedure. The bottom panels (C) represent the blanks of the above human serum and urine samples.

The chromatographic conditions used are modifications of two previously published reports [7,9]. In contrast to these reports a narrow-bore column was used (2 mm I.D.). The minimum detection level for PAH and IOT was 0.125 μg/ml and 0.250 μg/ml, respectively at a signal-to-noise ratio of 3:1. An alteration to the previous methods was the use of acetonitrile as organic phase, which led to an improved peak shape. The retention times for PAH, PABA and IOT at a flow-rate of 0.3 ml/min were 4.4, 5.4 and 12.4 min, respectively.

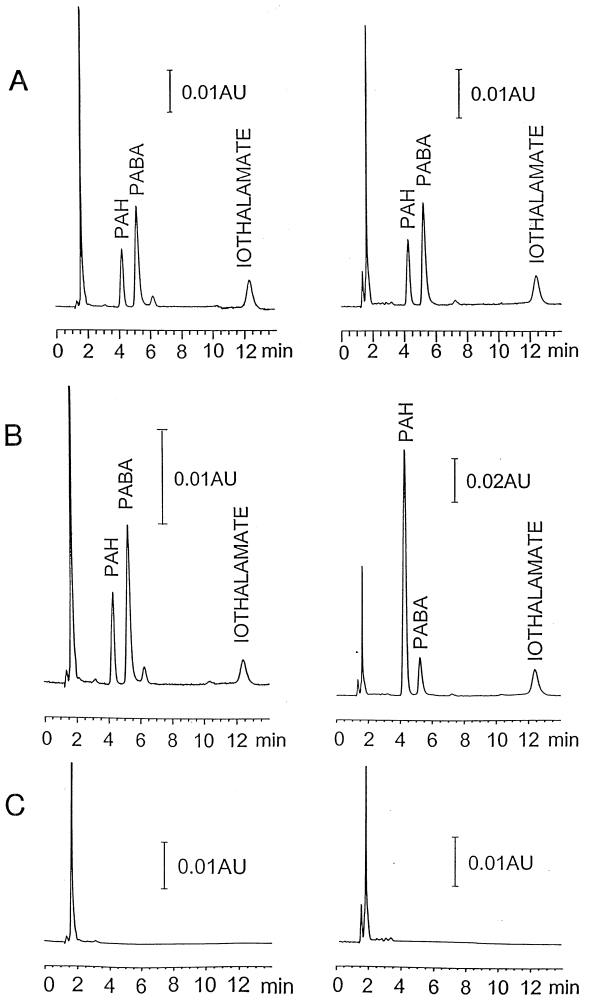

Fig. 1. (A) Standard chromatograms of supernatants (precipitation method) of serum (left panel) and urine samples (right panel), supplemented with PAH (serum and urine 25 μg/ml), IOT (serum and urine 50 μg/ml) and PABA (serum and urine 33.3 μg/ml). (B) Chromatograms of supernatants (precipitation method) of serum (left panel) and urine samples (right panel) derived from a patient undergoing evaluation of kidney function with PAH and IOT. (C) Chromatograms of the supernatants (precipitation method) of the corresponding serum (left panel) and urine samples (right panel) from the patient prior to drug infusion (blanks).

Table 1
Mean values, standard deviation and relative standard deviation for the four analysed concentrations of PAH and iothalamate in serum using precipitation (Prec) and ultrafiltration (Uf) methods

	Actual concentration (μg/ml)	Precipitation mean (μg/ml)	Ultrafiltration mean (μg/ml)	SD Prec (μg/ml)	SD Uf (μg/ml)	RSD Prec (%)	RSD Uf (%)
PAH serum	3.125	3.11	2.81	0.06	0.11	2.0	3.8
	6.25	6.19	5.51	0.10	0.09	1.6	1.6
	12.50	12.35	11.25	0.15	0.20	1.2	1.8
	25.00	24.36	22.15	0.50	0.25	2.0	1.1
Iothalamate serum	6.25	5.41	5.34	0.17	0.15	3.0	2.8
	12.50	11.28	10.94	0.14	0.18	1.2	1.7
	25.00	24.10	22.87	0.84	0.45	3.5	2.0
	50.00	48.78	46.49	1.11	0.43	2.3	0.9

For both sample preparation methods mean values, SDs and RSDs of PAH and IOT in serum and urine are shown in Tables 1 and 2, respectively. The regression coefficients of the calibration curves of PAH and IOT in serum and urine were 0.99 for both sample preparation methods.

In comparison acetonitrile precipitation vs. ultrafiltration recovery for PAH in serum was 99.5% vs. 89.8% and in urine 97.9% vs. 96.0%. IOT gave recoveries of 93.3% vs. 90% and 97.6% vs. 96.3%, in serum and urine, respectively.

The results obtained by both methods are similar. The acetonitrile precipitation method showed a slightly higher recovery in both serum and urine, which is not due to volume constriction (internal standard). In comparison only PAH in serum had a significantly higher recovery (99.5 vs. 89.8%) when the acetonitrile precipitation method was applied.

The acetonitrile precipitation method and the ultrafiltration procedure for sample preparation prior to HPLC analysis have been applied in clinical routine. As an example Table 3 shows the results of the determination of GFR and ERPF of a patient undergoing kidney function evaluation after obtaining an informed consent approved by the local Ethics Committee. The patient's kidney function was investigated on three different days (days 1, 2 and 7). The infusion protocol used was similar to that described by Agarwal et al. [11]. We were able to demonstrate in this study that continuous infusion with prostaglandin E1 which lowered blood pressure did not deteriorate kidney function [12].

The application of the filtration sample preparation method according to Lowe et al. [8] was primarily used in our clinical study. Due to the cost-effectiveness of the precipitation method originally utilized by Bell et al. [7] in rats and recently also used for the evaluation of kidney function in healthy human volunteers by Agarwal [11] we applied this method in our ongoing clinical studies. We therefore evalu-

Table 2
Mean values, standard deviation and relative standard deviation for the four analysed concentrations of PAH and iothalamate in urine using precipitation (Prec) and ultrafiltration (Uf) methods

	Actual concentration (μg/ml)	Precipitation mean (μg/ml)	Ultrafiltration mean (μg/ml)	SD Prec (μmg/ml)	SD Uf (μg/ml)	RSD Prec (%)	RSD Uf (%)
PAH urine	3.125	2.88	2.85	0.04	0.07	1.5	2.6
	6.25	5.91	5.77	0.14	0.07	2.4	1.2
	12.50	12.15	11.90	0.18	0.15	1.5	1.3
	25.00	24.65	24. 06	0.23	0.17	0.9	0.7
Iothalamate urine	6.25	6.20	5.84	0.22	0.15	3.5	2.5
	12.50	11.82	11.82	0.40	0.28	3.4	2.3
	25.00	24.43	24.50	0.35	0.54	1.4	2.2
	50.00	50.00	50.10	0.73	0.63	1.5	1.2

Table 3
Results of the determination of GRF and ERPF of a patient on three consecutive days using both preparation methods, filtration vs. precipitation

	Patient A					
	ERPF (ml/min)			GFR (ml/min)		
	PAH			Iothalamate		
	Filtration		Precipitation	Filtration		Precipitation
Day 1	92.0	vs.	94.5	36.9	vs.	41.2
Day 2	129.1	vs.	125.4	52.0	vs.	52.0
Day 7	137.5	vs.	140.0	58.6	vs.	60.3

ated the two different deproteinization procedures. Our results demonstrate the comparability of the two techniques and therefore allowed us to continue our follow-up investigations with the acetonitrile sample preparation method.

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