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High-performance liquid chromatography of the renal blood flow marker *p*-aminohippuric acid (PAH) and its metabolite N-acetyl PAH improves PAH clearance measurements

L.A. Decosterd[a,*], A. Karagiannis[1,b], J.-M. Roulet[c], N. Bélaz[a], M. Appenzeller[a], T. Buclin[a], P. Vogel[c], J. Biollaz[a]

[a]*Division de Pharmacologie Clinique, Département de Médecine, Centre Hospitalier Universitaire Vaudois 1011, Lausanne, Switzerland*
[b]*Division d'Hypertension, Département de Médecine, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland*
[c]*Institut de Chimie Organique, Faculté des Sciences, Université de Lausanne, Lausanne, Switzerland*

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Abstract

PAH (N-(4-aminobenzoyl)glycin) clearance measurements have been used for 50 years in clinical research for the determination of renal plasma flow. The quantitation of PAH in plasma or urine is generally performed by colorimetric method after diazotation reaction but the measurements must be corrected for the unspecific residual response observed in blank plasma. We have developed a HPLC method to specifically determine PAH and its metabolite NAc-PAH using a gradient elution ion-pair reversed-phase chromatography with UV detection at 273 and 265 nm, respectively. The separations were performed at room temperature on a ChromCart® (125 mm×4 mm I.D.) Nucleosil 100-5 μm C_{18} AB cartridge column, using a gradient elution of MeOH–buffer pH 3.9 1:99→15:85 over 15 min. The pH 3.9 buffered aqueous solution consisted in a mixture of 375 ml sodium citrate–citric acid solution (21.01 g citric acid and 8.0 g NaOH per liter), added up with 2.7 ml H_3PO_4 85%, 1.0 g of sodium heptanesulfonate and completed ad 1000 ml with ultrapure water. The N-acetyltransferase activity does not seem to notably affect PAH clearances, although NAc-PAH represents 10.2±2.7% of PAH excreted unchanged in 12 healthy subjects. The performance of the HPLC and the colorimetric method have been compared using urine and plasma samples collected from healthy volunteers. Good correlations (r=0.94 and 0.97, for plasma and urine, respectively) are found between the results obtained with both techniques. However, the colorimetric method gives higher concentrations of PAH in urine and lower concentrations in plasma than those determined by HPLC. Hence, both renal (Cl_R) and systemic (Cl_S) clearances are systematically higher (35.1 and 17.8%, respectively) with the colorimetric method. The fraction of PAH excreted by the kidney Cl_R/Cl_S calculated from HPLC data (n=143) is, as expected, always <1 (mean=0.73±0.11), whereas the colorimetric method gives a mean extraction ratio of 0.87±0.13 implying some unphysiological values (>1). In conclusion, HPLC not only enables the simultaneous quantitation of PAH and NAc-PAH, but may also provide more accurate and precise PAH clearance measurements.

Keywords: *p*-Aminohippuric acid; N-Acetyl-*p*-aminohippuric acid

*Corresponding author.
[1]On leave from: Aristotelion University of Thessaloniki, Ippokration General Hospital, Thessaloniki, Greece.

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1. Introduction

For over 50 years, *p*-aminohippuric acid (PAH) clearance has been widely used in clinical research for the determination of renal plasma flow (RPF). PAH is actively secreted in the proximal tubules as well as being filtered through the glomeruli. At concentration below 30 μg/ml, approximately ninety percent of PAH is removed after a single passage through the kidney. Clinically, the extraction ratio is generally assumed to equal one. It has therefore been accepted that the PAH clearance reflects the renal plasma flow and is calculated with plasma and urinary levels of PAH, neglecting the renal venous blood level [1].

However, the presence of the 4-aminophenyl structure in PAH suggests that this renal marker – by definition not expected to be cleared from the body by metabolism – may nevertheless undergo N-acetylation. Indeed, early clinical investigators did notice that some PAH was biotransformed and converted to a conjugate soon identified as the N-acetyl-PAH [2].

In humans, the N-acetyltransferase (NAT) activity is dependent on two highly similar NAT genes. Whereas NAT2, responsible for the N-acetylation of sulfamethazine, isoniazide and caffeine, has been shown conclusively to represent a polymorphic locus [3], NAT1 – which N-acetylates the less sterically hindered *p*-aminobenzoic acid (PABA) – was thought to encode a genetically invariant protein. However, the presence of discrete NAT1 structural variants has been recently demonstrated among Caucasians [4,5]. This polymorphism was also revealed in human tissues, notably bladder and colon [6].

It seems reasonable therefore to investigate whether this (NAT1- or NAT2-dependent) renal and/or extrarenal metabolism of PAH influences to an important extent the accuracy of RPF calculations.

Previous applications of HPLC to PAH analysis were restricted to the dosage of PAH only using the somewhat tedious dual-wavelength monitoring [7] and was notably used in one study comparing this chromatographic technique with the colorimetric method after diazotation reaction [8].

Recently, Prescott and his group reported the occurrence of the N-acetyl metabolite of PAH in plasma and urine of healthy subjects and patients with impaired renal function [9]. PAH and NAc-PAH were analysed [10] separately in two HPLC runs using different chromatographic columns with distinct technical characteristics (I.D. and packing material) and solvent systems. This approach requires duplicate samples, and probably makes the analysis more time consuming.

We have developed a simple, direct HPLC method for the simultaneous determination of PAH and its major metabolite NAc-PAH, using a gradient elution ion-pair reversed-phase chromatography, with UV–DAD detection at their respective maximum absorption wavelength. The performance of the HPLC approach has been compared with the colorimetric method with emphasis on its influence on PAH clearance determination.

2. Experimental

2.1. Chemicals

4-Aminohippuric acid [N-(4-aminobenzoyl)glycin, PAH, (**1** in Fig. 1) puriss. p.a., 4-acetamidobenzoic acid purum, N-acetyl-PABA, internal standard, (I.S.)], 1-heptanesulfonic sodium monohydrate puriss p.a., N-(1-naphtyl)ethylenediamine dihydrochloride puriss p.a. and sodium hydroxyde puriss. p.a. pellets were purchased from Fluka (Buchs, Switzerland). Citric acid monohydrate cryst. (ACS 'Baker Analysed' reagent) was obtained from JT Baker (Deventer, Netherlands). Ammonium amidosulfamate p.a.,

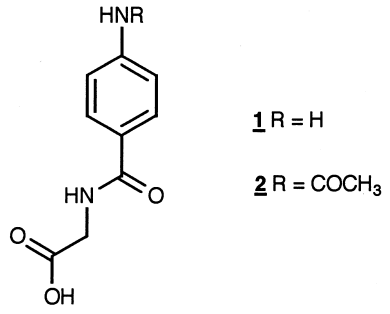

Fig. 1. Chemical structure of *p*-aminohippuric acid and N-acetyl-*p*-aminohippuric acid.

orthophosphoric acid (85%) p.a., acetonitrile (MeCN) gradient grade and methanol (MeOH) for chromatography LiChrosolv®, trichloroacetic acid (TCA) 20% solution were from E. Merck (Darmstadt, Germany). N-Acetyl-PAH (**2** in Fig. 1) was synthesised at the Laboratory of Organic Chemistry (Faculty of Sciences, University of Lausanne) (see below). All other chemicals were of analytical grade and used as received.

2.2. Chromatographic system

The chromatographic system consisted of a Hewlett–Packard 1090 (serie II) (Hewlett–Packard, Germany) equipped with a spectrophotometric UV–VIS photodiode-array detector (DAD) (4 nm slit) set at 273 nm for 5 min and subsequently switched at 265 nm up to the end of the 20 min run. The software HPChemStation A.02.05 loaded on an HP Vectra 486/33N (8M RAM) was used to pilot the HPLC instrument and to process the data (area integration, calculation and plotting of chromatograms) throughout the method validation and samples analysis. The mobile phase was delivered at 1 ml/min using a gradient elution of MeOH–buffer pH 3.9 1:99→15:85 over 15 min. Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore). Both solvents were degassed by sparging with helium.

The separations were performed at r.t. on a ChromCart® cartridge column (125 mm×4 mm I.D.) filled with Nucleosil 100 – 5 μm C_{18} AB (Macherey–Nagel, Düren, Germany) equipped with a guard column (8 mm×4 mm I.D.) filled with the same packing material. The injection volume was 25 μl in this study but could conveniently be increased to 100 μl.

2.3. Solutions

A sodium citrate–citric acid solution (solution C) was prepared: 21.01 g of citric acid monohydrate and 8.0 g of NaOH were dissolved in 1000.0 ml water. The buffered (pH 3.9) aqueous component of the mobile phase had the following composition: 1.0 g of sodium heptanesulfonate monohydrate dissolved in 375 ml of solution C, added up with 2.7 ml of orthophosphoric acid 85% and completed to 1000.0 ml with ultrapure water.

2.4. Stock solution, standard and control samples

2.4.1. Plasma calibration and control standard

Stock solutions were prepared by dissolving PAH (50 mg) in 50.0 ml of MeOH and NAc-PAH (5.0 mg) in 5.0 ml MeOH. Plasma calibration standards at 2.5–40 μg/ml of PAH and NAc-PAH together with plasma control samples at 12, 25, 35 μg/ml were prepared in batches of 10 ml by adding an appropriate volume of the respective stock solution to blank plasma from outdated transfusion bags (total added volume ≤10% of the biological sample volume). The six calibration standards and control samples were stored as 250 μl aliquots in polypropylene Eppendorf tubes at −25°C until use and thawed the day of the analysis.

2.5. Urine calibration and control standard

Concentrated stock solutions were prepared by dissolving PAH (125.0 mg) in 50.0 ml of MeOH and NAc-PAH (12.5 mg) in 5.0 ml MeOH. Urine calibration standards at 20–200 μg/ml and control samples at 40, 60, 110 μg/ml of PAH and NAc-PAH were prepared in batches of 10 ml by adding an appropriate volume of the respective stock solution to a blank urine pool (previously diluted 1:50 with water) obtained from healthy volunteers. The 1:50 dilution of the urine matrix was chosen to match the average dilution carried out with samples collected during the clinical studies. These standards were similarly stored as 250 μl aliquots at −25°C.

2.6. Internal standard solution

A concentrated (0.333 mg/ml) stock solution of internal standard (N-acetyl-PABA, 33.3 mg in 100.0 ml MeCN–H_2O, 1:9) was prepared prior to each batch of analysis and stored in the dark at 4°C. After equilibration at r.t., this solution was directly used for urine sample analysis. For plasma, this solution was diluted 1 part plus 2 parts ultrapure water prior to use (0.111 mg/ml).

2.7. Processing of samples

The urine samples collected during the clinical studies were diluted with ultrapure water to attain PAH concentrations lying within the range of concentrations of the calibration curve, according to the expected levels calculated from the rate of PAH infusion and the volume and collection time of urine.

One hundred microliters of calibration, control or patients' samples (plasma or diluted urine) were mixed and vortexed with 25 μl of the corresponding internal standard solution (0.111 and 0.333 mg/ml for plasma and urine, respectively) in an Eppendorf microvial. After the addition of 25 μl of TCA 20% the solution was vortexed and centrifuged (10 000*g*) for 10 min (Eppendorf 5413 Benchtop Centrifuge). The supernatant was introduced into a 200 μl HPLC microvial (Hewlett–Packard, Germany), and 25 μl of this solution was injected into the HPLC column. Increasing the injection volume to 100 μl resulted in an improvement of the reliability and of the inter-day variability of the method, as observed in a second series of analyses of samples collected for another study (see below).

2.8. Calibration curves

Quantitative analysis of PAH and NAc-PAH were performed using the internal standard (I.S., NAc-PABA) method. Calibration curves were obtained by unweighted least-squares linear regression analysis of the peak-area ratio of PAH and NAcPAH to I.S. versus the ratio of the injected amount of PAH, respectively, NAcPAH to I.S., in each standard solution. Over the range 2.5–40 μg/ml and 20–200 μg/ml for plasma and urine, the regression coefficient r^2 of the calibration curves for PAH and NAc-PAH in plasma and urine were good and always >0.997 (Table 1).

2.9. Analytical method validation

The validation of the method was performed according to the recommendations published as a Conference Report of the Washington Conference on Analytical methods validation: Bioavailability, Bioequivalence and Pharmacokinetic studies [11].

Each level of the calibration curve was measured with two sets of calibration standard samples: one set at the beginning and a second set at the end of the HPLC run. Throughout the biological sample analysis, control samples at three relevant concentration levels (12, 25 and 35 μg/ml, and 40, 60 and 110 μg/ml, for plasma and urine, respectively) were assayed every five samples. All samples (calibration, quality control and patients) were analysed in duplicate with the duplication process starting at the extraction step.

The quality control samples were used for the determination of the precision and accuracy of the method. The precision was calculated as the coefficient of variation (C.V.%) within a single run (intra-assay) and between different assays (inter-assays). Accuracy was determined as the percentage of deviation between nominal and measured concentration obtained from the established calibration curves.

The limit of quantification (LOQ) for PAH and NAc-PAH in plasma was chosen as the concentration of sample where the deviation between measured and nominal concentration does not deviate more than ±20% and was used for the lowest concentration level on the standard curve, in accordance with the

Table 1
Mean parameters of the calibration curves for PAH and NAc-PAH (internal standard method)

	$y=mx+b$		Regression coefficient (r^2)
	m	*b*	
Plasma (n=8)			
PAH	6.54E−01±0.030	2.30E−02±0.007	0.999±0.001
NAc-PAH	6.83E−01±0.016	1.90E−02±0.005	0.997±0.002
Urines (n=12)			
PAH	6.43E−01±0.017	2.89E−05±0.012	1.000±0.000
NAc-PAH	6.25E−01±0.014	3.40E−02±0.017	0.997±0.001

Conference Report on Analytical Methods validation [11].

The limit of detection (LOD) in plasma was determined by analysing spiked plasmas in duplicate with concentrations of PAH and NAc-PAH from 5 down to 0.1 μg/ml.

The stability of PAH and NAc-PAH was determined as follows:

(1) Aliquots of plasma and urine spiked with various (low, medium, high) concentrations of PAH and NAc-PAH underwent three freeze–thaw cycles: frozen triplicate samples were allowed to thaw at ambient temperature for one hour and were subsequently refrozen. The PAH and NAc-PAH levels were compared with aliquots not submitted to the freeze–thaw cycles.

(2) Processed plasma samples (i.e. containing PAH and NAc-PAH at low, medium and high concentration in TCA medium) were stored at room temperature for 24 h. The area of the signals of PAH and NAc-PAH were determined each hour by HPLC.

2.10. Isolation of the metabolite NAc-PAH

The isolation of NAc-PAH from a selected urine sample (collected from a subject having received i.v. PAH) was done by semi-preparative HPLC on the previously mentioned analytical column with UV detection at 230 nm using MeOH–AcOH 0.1% (5:95) and injection of 10 μl undiluted urine. A larger volume (25 μl) of undiluted urine could be injected with a slightly different solvent mixture (MeOH–0.1% AcOH, 3:97). The peak was collected, then evaporated on a water bath at 37°C in vacuo (Rotary Evaporator, Heidolph VV2001, Kelheim, Germany), dried and submitted to ^{1}H NMR and EI–MS spectroscopy.

2.11. Metabolite characterisation

The metabolite was characterised by nuclear magnetic resonance spectroscopy (^{1}H NMR, Bruker ARX-400) and by mass spectroscopy by electron spray ionisation (ESI–MS, Finnigan MAT SSQ 710C).

^{1}H NMR (400 MHz, CD_3CN): 8.53 (*s*, –NH–); 7.78 (*m*, H *arom.*); 7.66 (*m*, H *arom.*); 4.02 (*d*, ^3J(H–C; –NH–)=6.2 Hz, $-H_2C-$); 2.11 (*s*, CH_3CO). ESI–MS (CH_3CN): *m/z* 237.4.

The identity of compound **2** (Fig. 1) isolated from urine was confirmed by comparison with synthetic NAc-PAH (^{1}H NMR, retention time on RP-HPLC).

2.12. Synthesis of NAc-PAH

One gram (5 m*M*) of PAH was dissolved in 5 ml HCl 5%. 1 *M* NaOH was added until basic pH was attained. A few chips of ice were then added, followed by 10 ml of acetic anhydride. The mixture was stirred, and a solution of 10 g sodium acetate in 10 ml H_2O was added in one portion. After 15 min, the solution was acidified and then extracted with AcOEt (3×10 ml). The organic layer was dried over $MgSO_4$ and evaporated in vacuo. The residue was recrystallised from MeOH, affording 1.05 g of NAc-PAH (yield: 86%).

2.13. Colorimetric measurement of PAH after diazotation reaction

The colorimetric method used is a microadaptation of the Bratton–Marshall procedure [12,13]. Briefly, the plasma is deproteinised with 10% TCA (1 part of plasma+4 parts of 10% TCA) and centrifuged 20 min (Eppendorf 5413 Benchtop Centrifuge). The urines are diluted with 10% TCA to attain the desired 0–40 μg/ml concentration range. Aliquots (100 μl) of standard solutions of PAH diluted in 10% TCA (up to 40 μg/ml), deproteinised plasma and diluted urines are mixed with 200 μl $NaNO_2$ (1 mg/ml). After 5 min, 200 μl of $NH_2SO_3NH_4$ (5 mg/ml) are added and the solution is allowed to stand for a further 5 min period. The coupling reagent [500 μl of a 1 mg/ml solution of N-(1-naphtyl)ethylenediamine] is added and the resulting solution is vortexed and measured spectrophotometrically at 546 nm after 30 min – but within 2 h – against blank solutions containing all reagents. The residual unspecific value (observed in blank plasma from each subject) is subtracted from all subsequent analysis.

2.14. PAH clearance

According to a protocol previously approved by the Ethics Committee of the Hospital, twelve healthy male volunteers received a priming dose of PAH followed by a constant rate infusion aimed at achiev-

ing and maintaining plasma concentrations of approximately 20 μg/ml. At predetermined times, blood samples were drawn into 4 ml Monovettes® tubes (Sarstedt, Nürnberg, Germany) containing EDTA-K and immediately centrifuged for 10 min at 4°C. Similarly, at scheduled time, urine was collected and its volume measured. Plasma and urine samples were stored at −25°C prior to analysis.

The systemic clearance (Cl_S), i.e. the clearance by all eliminating organs, was calculated as the infusion rate (R_{in}) (mg/min) divided by the plasma concentration (mg/ml) at steady state (C_{SS}) and is expressed in ml/min. The renal clearance (Cl_R) was determined as the ratio of the excretion rate of PAH in the urine [urine concentration (U)×urine flow (V) in mg/min] divided by the average of the plasma concentration P (mg/ml) obtained at the beginning and at the end of each urine collection. The fraction of PAH excreted by the kidney is the ratio of the renal and the systemic clearances (Cl_R/Cl_S). The clearance to the metabolite (Cl_{MET}) was obtained by dividing the urinary excretion of NAc-PAH (mg/min), expressed as the corresponding PAH amount (R_{out}), by the plasma concentration of PAH. The concordance of colorimetric vs. HPLC measurements was assessed according to the proposal of Lin [14]. In brief, the correlation coefficient between both measures is multiplied by a coefficient of congruence, derived from the scale and location shifts between the measures. The resulting number is an estimate of the concordance between the measures.

3. Results

3.1. Chromatograms

The proposed HPLC method provides a simple procedure to quantify simultaneously PAH and NAc-PAH in a single run. Fig. 2c shows the chromatogram of a plasma sample from one volunteer at steady state. With the gradient program used, the peaks of PAH, NAc-PAH and NAc-PABA (I.S.) appear at 3.6, 7.8 and 12.3 min, respectively. No interfering peaks are detected at the same retention time as shown in plasma either blank (Fig. 2a) or spiked with IS, showing notably that NAc-PABA is stable in the experimental conditions (Fig. 2b). The chromatographic profile of a urine sample, either blank or spiked with I.S., and a urine sample obtained from the same volunteer at steady-state (urine collected 2–4 h after starting the constant rate infusion) are shown in Fig. 2d–f, respectively.

3.2. Calibration curves

The standard curves for PAH and NAc-PAH were satisfactorily described by unweighted least-squares linear regression analysis over the concentration range 2.5–40 μg/ml in plasma and 20–200 μg/ml in urine. Mean standard curve parameters obtained throughout the analyses of these series of sample are reported in Table 1. Both the parameters of the curves and the intercepts were stable throughout the two months analysis. In all cases the mean regression coefficients were $0.997<r^2<1.000$ either for the plasma or urine calibration curves.

3.3. Validation of the HPLC method: precision, accuracy, LOQ and LOD

The precision and accuracy of the control samples for these series of analysis are given in Table 2 and Table 3. The levels of concentration of the PAH control samples at 12, 25 and 35 μg/ml were selected to encompass the 15–35 μg/ml range of concentrations presumably present in plasma collected in this clinical study with healthy volunteers (see Fig. 3a). Similarly, the concentrations of the urine control samples at 40, 60 and 110 μg/ml were chosen according to the expected levels calculated from the rate of PAH infusion and the volume and collection time of urine of the subjects.

Throughout these relevant concentration ranges, the mean intra-assay precision was 2.9 and 3.6% for PAH and 2.7 and 3.6% for NAc-PAH, in plasma and urine, respectively. Overall, the mean inter-day precision was slightly higher with mean CV values comprised within the range 2.0–4.3%. In urine, intra- and inter-assay accuracy were acceptable with a deviation never exceeding −12.5%. In plasma, the intra-assay accuracy measured for PAH and NAc-PAH were good (≤11.8%) and the mean inter-day deviation of the control samples of NAc-PAH always <15%. While there is no valid explanation for the large inter-day deviation of PAH plasma control at

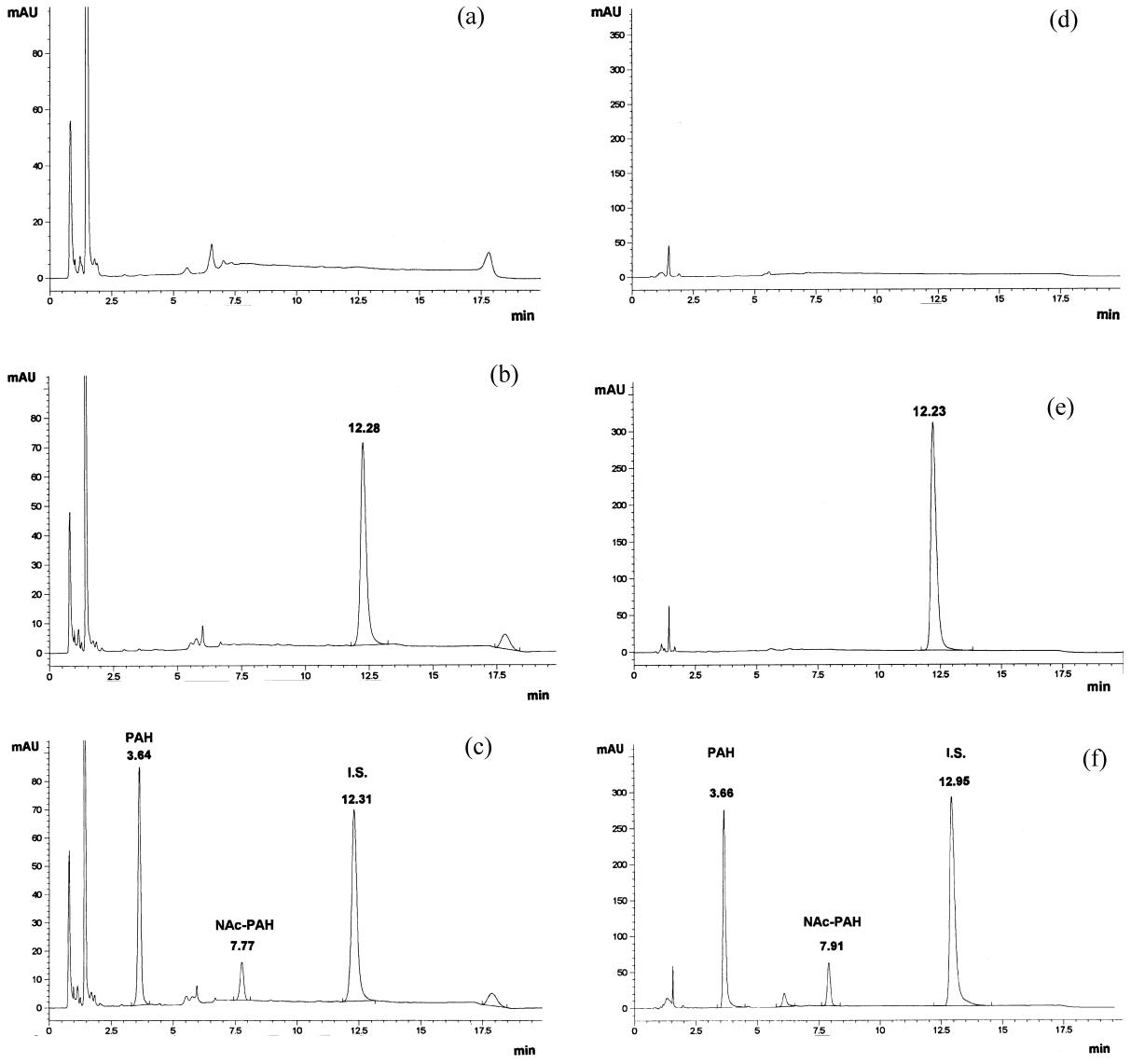

Fig. 2. Chromatograms obtained from (a) blank plasma, (b) blank plasma spiked with NAc-PABA (I.S.), (c) plasma from one volunteer at steady state (with PAH and NAc-PAH at the concentration of 23.3 and 4.4 μg/ml, respectively), (d) blank urine, (e) blank urine spiked with NAc-PABA (I.S.) (dilution factor 62 times), (f) diluted urine of the same volunteer at steady state (with PAH and NAc-PAH at the concentrations of 59.8 and 11.1 μg/ml, respectively) corresponding to concentrations of 3708 and 688 μg/ml for PAH and NAc-PAH, respectively, in undiluted urine collected 2–4 h after starting the constant rate infusion.

12 μg/ml which exceptionally reached 19% in these first series of analysis, this value could however be improved by increasing from 25 to 100 μl the volume of sample injected onto the HPLC column. This aforementioned modification was indeed applied for the analysis of samples collected in another study (Biollaz et al., in preparation) which resulted in inter-assay deviation values for plasma control samples of PAH at 12 μg/ml lower than −12.2% and produced an improvement of the overall inter-assay values. In these latter series of analysis, the concentration of the plasma control at the highest NAc-

Table 2
Precision and accuracy of the HPLC assay for PAH and NAc-PAH in plasma

PAH				N-Acetyl PAH			
Nominal conc. (μg/ml)	Conc. found (μg/ml)	Precision C.V. %	Accuracy[a] Deviation (%)	Nominal conc. (μg/ml)	Conc. found (μg/ml)	Precision C.V. %	Accuracy[a] Deviation (%)
A. Intra-assay (n=12)							
35.11	33.8±0.9	2.7	−3.6	35.0	34.7±0.7	2.1	−1.0
25.08	23.6±0.7	3.0	−6.1	25.0	23.5±0.7	3.0	−6.1
12.04	12.6±0.4	3.0	4.4	12.0	10.6±0.3	3.0	−11.8
B. Inter-assay (n=13)							
35.1	36.7±1.2	3.3	4.5	35.3	40.5±0.9	2.3	14.8
25.1	25.1±1.3	5.3	0.1	25.2	28.0±1.3	4.8	10.9
12.0	14.3±0.6	4.4	19.0	12.1	13.8±0.4	3.0	14.0

[a] (Found−Nominal)/Nominal×100.

PAH concentration was reduced from 35 μg/ml (highly unlikely to appear in clinical samples at the administered dose of PAH, as observed a posteriori in plasma from healthy subjects) to 25 μg/ml. Here again, the inter-assay ($n=20$) precision and accuracy values for control samples at 12, 18 and 25 μg/ml of NAcPAH were always lower than 5.6%.

By analysing spiked plasmas at concentrations from 5 down to 0.1 μg/ml, the limit of detection (LOD) in plasma was experimentally found to be 0.2 μg/ml for both PAH and NAc-PAH. The determination of the LOD in urine was not considered since there is no analytical limitations to measure by HPLC the relatively high (at the mg/ml range) concentrations of PAH/NAc-PAH in urine.

At the lowest considered PAH and NAcPAH concentrations in plasma (2.5 μg/ml) the deviation between nominal and measured values were −19.4 and −17%, respectively. This concentration was therefore chosen for the lowest concentration level on the plasma standard curve, in accordance with the recommendations [11] stating that the accuracy of sample at the LOQ (limit of quantification) must not exceed ±20%.

3.4. Samples stability

When subjected to three freeze–thaw cycles, control plasma and urine samples of PAH/NAc-PAH at the three concentration levels were found to be stable taking into account the experimental variability. The stability of PAH/NAc-PAH (at 12, 25

Table 3
Precision and accuracy of the HPLC assay for PAH and NAc-PAH in urine

PAH				N-Acetyl PAH			
Nominal conc. (μg/ml)	Conc. found (μg/ml)	Precision C.V. %	Accuracy[a] Deviation (%)	Nominal conc. (μg/ml)	Conc. found (μg/ml)	Precision C.V. %	Accuracy[a] Deviation (%)
A. Intra-assay (n=12)							
109.6	95.9±2.6	2.7	−12.5	108.2	112.6±2.2	1.9	4.0
59.8	62.4±3.1	5.0	4.4	59.0	64.8±3.6	6.0	9.7
39.8	40.0±1.2	3.0	0.4	39.4	41.4±1.2	3.0	5.2
B. Inter-assay (n=19)							
109.6	101.2±2.0	2.0	−7.6	108.2	116.6±1.3	1.1	7.7
59.8	61.9±1.3	2.1	3.5	59.0	63.9±1	2.0	8.3
39.8	38.0±1.1	2.8	−5.5	39.4	40.0±1	3.0	2.7

[a] (Found−Nominal)/Nominal×100.

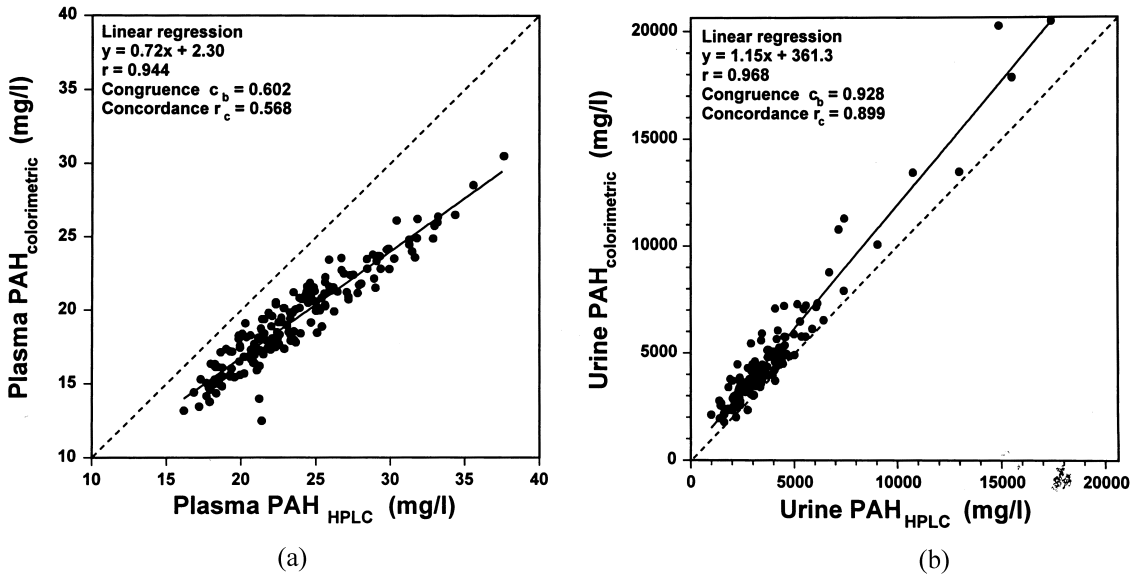

Fig. 3. (a) Plasma concentration of PAH measured by HPLC vs. colorimetry (n=180) (dotted line=identity line). (b) Urine concentration of PAH measured by HPLC vs. colorimetry (n=143), c_b=congruence coefficient; r_c=concordance coefficient.

and 35 μg/ml) in processed (containing TCA) samples submitted to HPLC analysis was also checked: after the removal of proteins from plasma, the concentration variation over time, expressed in percentage of the starting concentrations of PAH and NAc-PAH, was below 5% in the resulting acidic samples let at room temperature for 24 h. These observations indicate therefore that the stability of processed plasma samples was acceptable throughout their HPLC analysis.

The stability of plasma spiked with synthetic NAc-PAH at 12.1, 25.2 and 35.3 μg/ml and urine spiked at 39.4, 59.0 and 108.2 μg/ml was also assessed by colorimetry. The colorimetric response observed after 30 min was negligible, indicating that NAc-PAH is stable in 10% TCA under the described conditions and does not generate PAH since no diazonium ion is formed within the time of the colorimetric analysis.

3.5. Specificity of HPLC vs. colorimetric method

The precipitation step applied to plasma (1 part 20% TCA+4 parts plasma) before injecting it into the HPLC eliminates adequately all early eluting components. No interferences with the specific measurement of PAH are observed in the chromatogram of a blank plasma (Fig. 2a,b). By comparison, the assay of blank plasmas (n=36) taken from the 12 volunteers give non-specific responses with the colorimetric method, yielding PAH concentrations of 1.25±0.55 μg/ml which have therefore to be subtracted.

Good correlations (r=0.94 and 0.97) are observed between both techniques for plasma and urine (Fig. 3a,b). However, the colorimetric method gives consistently higher PAH concentrations in urine and lower concentrations in plasma when compared with HPLC. Since the difference does not appear to be caused by a systematic bias – the tendency is opposite in urine and plasma –, the matrix effect, not taken into account with the colorimetric method, may nevertheless influence the optical density.

3.6. Measurement of PAH clearance

Since the colorimetric method gives reproducibly higher PAH concentrations in urine (Fig. 3b) while the plasma concentrations are constantly lower (Fig. 3a), both renal and systemic clearances are systematically higher with the colorimetric than with the

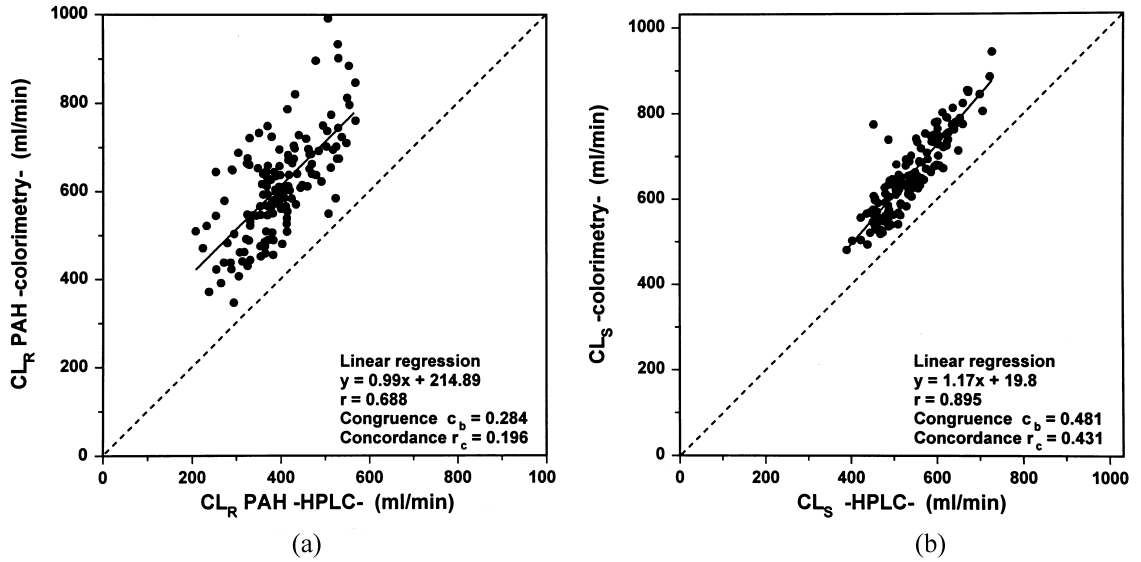

Fig. 4. (a) Renal clearance (CL_R) of PAH measured by HPLC vs. by colorimetry. (b) Systemic clearance (CL_s) of PAH measured by HPLC vs. colorimetry. c_b=congruence coefficient; r_c=concordance coefficient.

HPLC method (Fig. 4a,b) with accordingly mediocre concordance coefficients.

In our study, the mean clearance to the metabolite CL_{MET} was 33.1±7.6 ml/min and contributed little to the total clearance of PAH. It can be concluded that NAc-PAH formation is not clinically important and does not affect to a significant extent the use of PAH clearance for the determination of renal plasma flow.

When analysed by HPLC, the renal clearance

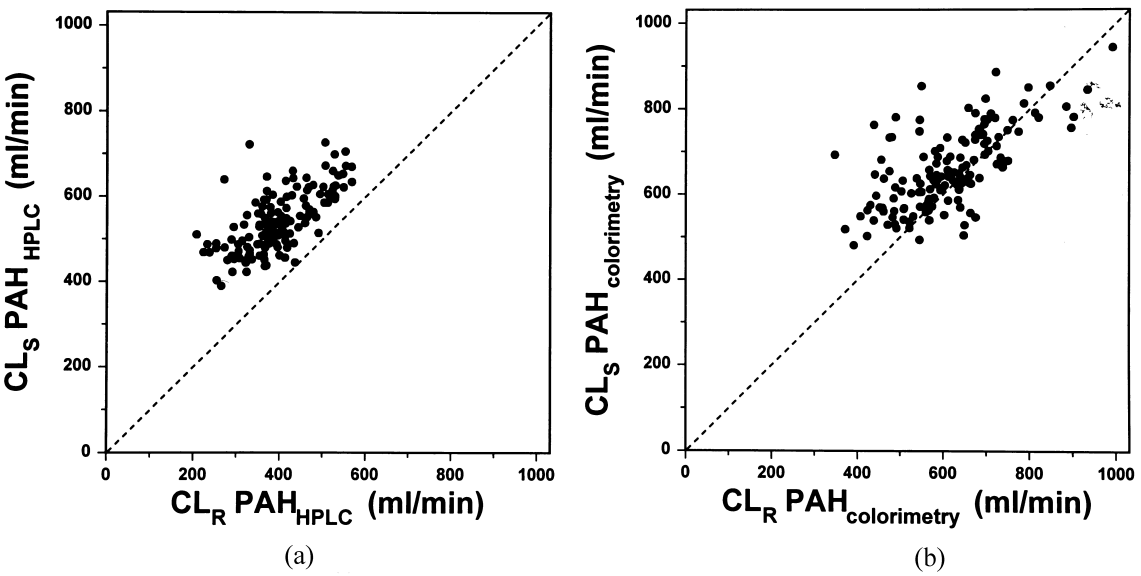

Fig. 5. (a) Renal clearance (CL_R) vs. systemic (CL_s) clearance of PAH measured by HPLC. (b) Renal clearance (CL_R) vs. systemic (CL_s) clearance of PAH measured by the colorimetric method.

(CL_R) of PAH is, as expected, always smaller than the systemic clearance CL_S. The fraction of PAH excreted by the kidney CL_R/CL_S is therefore always <1 (mean$=0.73\pm0.11$, $n=143$) (Fig. 5a). When determined by the colorimetric method, the mean extraction ratio is 0.87 ± 0.13, implying unphysiological values in some cases (ratio>1; cluster of points under the identity line) (Fig. 5b).

4. Discussion

4.1. Analytical method

Previous RP-HPLC methods for the assay of PAH proposed either cationic ion-pair agent such as tetrabutylammonium phosphate at pH 2.8 [7] or no ion-pair agents at all [8,10] in acidic mobile phase.

The zwitterion PAH is present in acidic pH in the cationic form and sodium heptanesulfonate (previously used for the assay of PABA and its urinary conjugates, among them PAH itself [15]), was chosen to retain PAH on the reversed-phase column long enough to prevent interferences from early eluting peaks. As expected with charged molecules, careful control of the pH of the mobile phase was crucial in obtaining reproducible retention times for the analytes. Automatic switching of the wavelength from 273 to 265 nm after 5 min allowed the optimal detection of PAH and NAc-PAH throughout the routine analysis. However, a simpler UV-detector set at one single wavelength (ca. 270 nm) could conveniently be used instead.

With the UV detection, the polyfructosan sinistrin, another renal exogenous marker used for glomerular filtration rate (GFR) determination [16], generally co-administrated with PAH, perturbed the detection of neither PAH nor NAc-PAH even at concentrations as high as 250 μg/ml in plasma and in the mg/ml range in urine.

4.2. N-Acetylation of PAH

The amount of NAc-PAH in urine corresponds to $10.2\pm2.7\%$ of the PAH excreted unchanged in 12 healthy subjects. Our results are similar though slightly lower than the $15.2\pm1.8\%$ previously reported by Prescott et al. in their ''step-up'' and ''step-down'' infusion administrations of PAH or with the $16.9\pm2.4\%$ of the total amount recovered in urine as NAc-PAH after the administration of PAH as a single bolus injection to male or female healthy subjects [9]. Interestingly in their study, the percentage of urinary NAc-PAH in patients with chronic renal failure after bolus injection of PAH was significantly higher with a mean recovery of $26.9\pm4.8\%$, albeit with a lower mean eight-hour total recovery of the dose when compared to healthy volunteers ($83.6\pm7.1\%$ vs. $102\pm9.4\%$, respectively).

As far as clinical investigations of the renal function are concerned, since the mean clearance to the metabolite CL_{MET} (33.1 ± 7.6 ml/min) contributed little to the total clearance of PAH (mean $Cl_s=541\pm71$ ml/min in our study, calculated with HPLC data), the NAT1- or NAT2-dependent N-acetylation of PAH does not seem clinically relevant nor does it notably affect PAH clearances and hence renal blood flow determinations.

4.3. Comparative study of PAH determination by HPLC vs. the colorimetric method

Using spiked plasma and urine samples at only one single fixed concentration, Kigushi et al. [8] concluded that the HPLC and colorimetric method [with a slightly different coupling reagent, i.e. 1-(β-diethylaminoethyl)-α-naphtyramine oxalate] was applicable to routine PAH determination (analytical error rate $<10\%$), stressing however that, next to the higher sensitivity of HPLC, the conditions (time and temperature) of the coupling reaction had a critical influence on the colorimetric response.

A comparison of both techniques has been performed in our hand using a total of 323 (143 urines and 180 plasmas) samples obtained from healthy volunteers. In spite of good correlations between the results obtained with both techniques, both renal and systemic clearances of PAH, calculated with the colorimetric data, are higher (35.1 and 17.8%, respectively) than those derived from the specific HPLC measurements, resulting in relatively mediocre concordance coefficients. These differences cannot be explained by the presence of NAc-PAH which did not react in the diazotation reaction under the conditions of our colorimetric assay. Unspecific responses due to the components of the biological

matrices are probably involved. When individual PAH clearances derived from colorimetry data were compared, some unphysiological values (i.e. renal clearance>systemic clearance) were obtained. Conversely, specific determination of PAH by HPLC lead to less dispersion of the clearances, and no unphysiological values.

The reported HPLC method enables the simultaneous quantitation of PAH and NAc-PAH. Further studies are still required to elucidate the site of the NAT1- or NAT2-dependent enzymes involved in this metabolism. Whereas the N-acetylation of PAH has probably little relevance to the estimation of the renal clearance of PAH as the standard measure of the effective renal plasma flow, it appears nevertheless that the HPLC provides more accurate and precise PAH clearance measurements. The reported high intraindividual variability of the renal blood flow may be due to the analytical limitations of the colorimetric method. Although more time-consuming and expensive, this HPLC method may nevertheless represent a valuable tool for the detection of minute changes of PAH clearances and hence of renal blood flow, in the clinical investigations of renal alterations induced by drugs and diseases.

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