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Short communication

Simple HPLC–UV method for determination of iohexol, iothalamate, *p*-aminohippuric acid and *n*-acetyl-*p*-aminohippuric acid in human plasma and urine with ERPF, GFR and ERPF/GFR ratio determination using colorimetric analysis

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Abstract

A simple high-performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of iohexol, iothalamate, *p*-aminohippuric acid (PAH) and *n*-acetyl-*p*-aminohippuric acid (*n*-acetyl-PAH) in human plasma and urine. A C_{18} column at a flow rate of 1 ml/min with an aqueous mobile phase of trifluoroacetic acid (0.1% TFA in deionized water (pH 2.2), v/v) and methanol gradient was used for component separation. The plasma and urine assay demonstrated linearity from 10 to 50 μg/ml for iohexol and iothalamate, 5 to 40 μg/ml for PAH and 2.5 to 40 μg/ml for *n*-acetyl-PAH. The HPLC plasma and urine results obtained for PAH were used to calculate the subject kidney effective renal plasma flow (ERPF) and the iohexol results were used to calculate the subject kidney glomerular filtration rate (GFR). The HPLC results for PAH were then compared to an alternative colorimetric method for analyzing PAH to determine if subject metabolism (acetylation) of PAH affected the ERPF results obtained using the colorimetric method, the subsequent ERPF/GFR ratio and clinical impression of subject patient kidney function. The method was utilized in several different clinical studies evaluating the effect of kidney function from medications (phase IV evaluations) marketed for patients with cardiovascular disease.

Keywords: Iohexol; Iothalamate; *p*-Aminohippuric acid (PAH); *n*-Acetyl-PAH; ERPF; GFR; HPLC

1. Introduction

Iohexol and iothalamate are currently indicated for use in GFR evaluation and *p*-aminohippuric acid (PAH) is indicated for use in effective renal plasma flow (ERPF) evaluation on patients diagnosed with renal disease [1–3]. Current published methods [4–12] utilize high-performance liquid chromatographic (HPLC) and various sample preparations (e.g. chloroform extraction [7]) and mobile phase additives (e.g. tetrabutylammonium sulphate [4,12]); however none offers a fast and simple simultaneous determination of iohexol, iothalamate, PAH and *n*-acetyl-PAH in human plasma and urine. The method detailed in the present communication utilizes a simple sample preparation for plasma and urine samples and does not require the use of an internal standard or ion pairing reagent for PAH measurement. In addition, this method employs current HPLC bioanalytical column technology, which provided sufficient component resolution and sensitivity for simultaneous measurement of these components in human plasma and urine. Plasma and urine PAH results were obtained using both HPLC and colorimetric [13] analysis to determine the ERPF and ERPF/GFR ratio. The HPLC plasma and urine results obtained for PAH were used to calculate patient kidney ERPF and then compared to colorimetric results on the same patient samples to deter-

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mine if metabolism (acetylation) of PAH affected ERPF results.

2. Experimental

2.1. Chemicals

PAH was purchased from Sigma (St. Louis, MO, USA). Iohexol (Omnipaque™) was purchased from Amersham Health (South Plainfield, NJ, USA) and Iothalamate Meglumine (Conray® 30%, U.S.P.) was purchased from Mallinckrodt Inc. (St. Louis, MO, USA) and both were used as received. The *n*-acetyl-PAH was prepared in-house using the published procedure [14] and determined to be 99% pure using HPLC. Trifluoroacetic acid was reagent grade, methanol and acetonitrile were both Optima HPLC grade and all were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure distilled and deionized water (18 megaohm) was prepared in-house and filtered prior to use.

2.2. HPLC equipment and mobile phase

The HPLC equipment consisted of a Hewlett-Packard (HP) Model 1090 HPLC (Agilent Technologies, Palo Alto, CA, USA). The analytical column was a Supelco Discovery® C_{18}, 250 mm × 4 mm i.d., 5 μm packing, 180 Å (Supelco, Bellefonte, PA, USA). The C_{18} guard column, 30 mm × 4.6 mm i.d., 40–50 μm pellicular packing (Alltech, Deerfield, IL, USA) was replaced prior to each analytical run, which typically consisted of approximately 75 samples. The mobile phase consisted of aqueous trifluoroacetic acid (0.1% TFA in deionized water (pH 2.2), v/v) and methanol gradient. The mobile phase gradient was linear with time course as follows (95:5 0.1% TFA in deionized water:methanol (v/v) at 0 min; 70:30 0.1% TFA in deionized water:methanol (v/v) at 12 min; 10:90 0.1% TFA in deionized water:methanol (v/v) at 13 min and held 3 min, and 95:5 0.1% TFA in deionized water:methanol (v/v) at 17 min).

The mobile phase was constantly degassed using helium sparging and used at a flow-rate of 1.0 ml/min. Typical HPLC operating pressure was approximately 150 bar at a column oven temperature of 40 °C. An injection volume of 5 μl of the prepared urine sample and 10 μl of the prepared plasma sample was accomplished using the HP Model 1090 autosampler. Component detection was achieved using the HP Model 1090 UV detector with an absorbance wavelength of 254 nm. The detector operated at high sensitivity setting with a 1 s response time. A 345 kPa back-pressure regulator (SSI, State College, PA, USA) was coupled to the detector outlet to prevent mobile phase outgassing. Data acquisition and component computations were performed using TotalChrom Workstation software (Perkin-Elmer™, Norwalk, CT, USA).

2.3. Standard and control preparation

Stock standards of PAH (180 mg/ml in 50:50 methanol:deionized water, v/v), *n*-acetyl-PAH (1.2 mg/ml in methanol), iohexol (388 mg/ml in deionized water) and iothalamate (300 mg/ml in deionized water) were prepared and stored at 4 °C. Working plasma and urine standards and controls of PAH (5–40 μg/ml), *n*-acetyl-PAH (2.5–40 μg/ml), iohexol (10–50 μg/ml) and iothalamate (10–50 μg/ml) were prepared using blank human plasma and urine as the diluents. All working standards and controls were stored and maintained at −20 °C, along with the study subject samples.

2.4. Sample conditions

Blood samples from subjects were collected at predetermined time-points according to study protocols in tubes containing heparin. Sample tubes were centrifuged at 3500 × *g* rpm for 15 min with plasma drawn off and frozen at −20 °C until analysis. Prior to analysis, plasma samples were thawed to ambient temperature, mixed thoroughly by inversion and centrifuged at 800 g for 10 min to eliminate fibrinous material. Urine samples (collected without preservative and stored at −20 °C) were thawed to ambient temperature, mixed thoroughly by inversion and allowed to sit 15 min for particulate matter to settle out.

2.5. Sample preparation

Plasma samples were prepared by pipetting 250 μl of plasma and 250 μl 0.1% TFA in deionized water into a polypropylene bullet centrifuge tube. Plasma proteins were precipitated by vortexing for 15 s. The samples were centrifuged at 13,000 × *g* for 10 min. The clear supernatant was transferred to glass HPLC autosampler vials. Urine samples were prepared by pipetting 20 μl of urine and 980 μl of deionized water directly into the glass HPLC autosampler vial and vortex mixing for 10 s. For urine and plasma sample analysis, 5 and 10 μl were injected into the HPLC system, respectively.

3. Results and discussion

3.1. Method optimization

The mobile phase aqueous component 0.1% TFA in deionized water was chosen as pH (∼2.2) provided good peak shape for PAH (pK_a 3.6) and eliminated the need of an ion pairing reagent (e.g. tetrabutylammonium chloride). The mobile phase organic modifiers (e.g. acetonitrile versus methanol) were evaluated to determine which organic solvent would provide the best chromatographic separation for these four components from endogenous plasma and urine components. When evaluating acetonitrile at various concentration levels in the mobile phase gradient, the iohexol

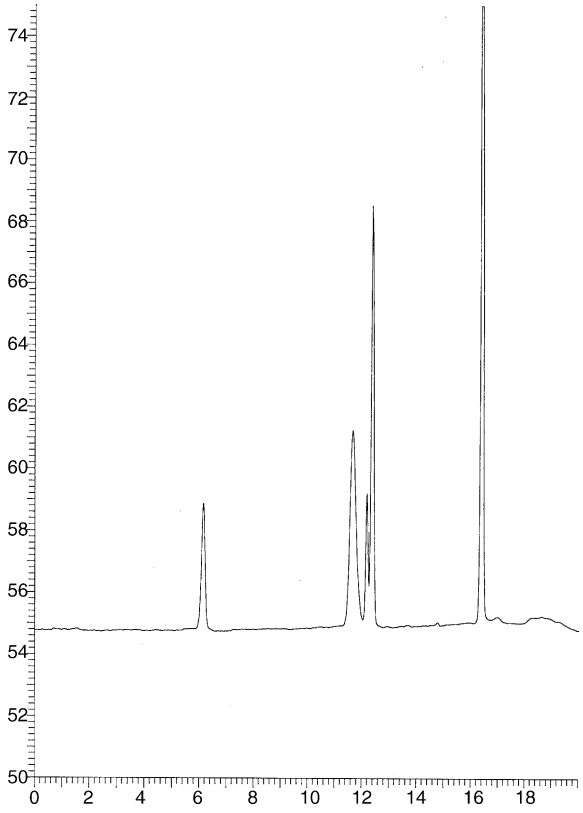

Fig. 1. Chromatogram illustrating iohexol isomers separation at 20 °C. The retention time of PAH (6.2 min), iothalamate (11.6 min), iohexol isomers (12.2 and 12.5 min) and *n*-acetyl-PAH (16.4 min). An HPLC column oven temperature of 40 °C will cause the iohexol isomers to co-elute for improved quantification and sensitivity of iohexol.

and iothalamate peak shape was found to be broader (larger peak width and peak tailing) which indicated reduced analytical column selectivity than observed using methanol as the organic modifier. Methanol was chosen as the organic modifier as it provided good component peak shape and selectivity from other endogenous components from the sample preparations. Different column oven temperatures (e.g. 20–50 °C) were evaluated with 40 °C found to be optimal for component peak shapes, aiding in the co-elution of the iohexol isomers (Fig. 1, [15]) for increased sensitivity and in lowering the HPLC system backpressure.

3.2. Linearity, limit of quantitation and detection, and computations

The plasma method was linear throughout the concentration range of 5–40 μg/ml for PAH (mean correlation coefficient of 0.9995, $n = 20$), 10–50 μg/ml for iohexol (mean correlation coefficient of 0.9993, $n = 20$), 10–50 μg/ml for iothalamate (mean correlation coefficient of 0.9990, $n = 4$), and 2.5–40 μg/ml for *n*-acetyl-PAH (mean correlation coefficient of 0.9998, $n = 20$). The urine method was linear throughout the concentration range of 5–40 μg/ml for PAH (mean correlation coefficient of 0.9990, $n = 20$), 10–50 μg/ml for iohexol (mean correlation coefficient of 0.9990, $n = 20$), 10–50 μg/ml for iothalamate (mean correlation coefficient of 0.9990, $n = 4$), and 2.5–40 μg/ml for *n*-acetyl-PAH (mean correlation coefficient of 0.9999, $n = 20$). The limit of detection for each component of the method was ∼1 μg/ml and was determined using a spiked amount of each component in each matrix at 1 μg/ml ($n = 3$) and calculated from standard curves for plasma and urine. For plasma and urine component calculations, normal linear regression utilizing external standardization and peak area was used. The lowest standard calibrator for plasma and urine was used as the limit of quantitation for reporting calculated subject results.

Table 1
Combined intra- and inter-day accuracy and precision for plasma controls

Component	Fortified concentration (μg/ml)	Calculated mean concentration (μg/ml)	%Error	%R.S.D.
PAH[a]	7.5	7.1	−5.3	12.1
PAH[a]	15.0	15.1	0.7	13.9
PAH[a]	35.0	34.2	−2.3	11.6
n-Acetyl-PAH[a]	7.5	7.4	−1.3	3.2
n-Acetyl-PAH[a]	15.0	14.8	−1.3	5.5
n-Acetyl-PAH[a]	30.0	29.2	−2.7	4.2
Iohexol[a]	15.0	14.9	−0.7	7.8
Iohexol[a]	25.0	24.8	−0.8	6.0
Iohexol[a]	45.0	43.6	−3.1	4.8
Iothalamate[b]	15.0	14.5	−3.3	8.0
Iothalamate[b]	25.0	26.8	7.2	10.0
Iothalamate[b]	45.0	43.2	−4.0	7.6

[a] $n = 75$.
[b] $n = 10$.

3.3. Accuracy, precision, and recovery

The accuracy and precision for the method was determined by evaluation of replicate prepared plasma (Table 1) and urine (Table 2) control samples. The intra-day (within day) and inter-day (between day) accuracy of the method was reported as the percent error of nominal spiked amounts ver-

Table 2
Combined intra- and inter-day accuracy and precision for urine controls

Component	Fortified concentration (μg/ml)	Calculated mean concentration (μg/ml)	%Error	%R.S.D.
PAH[a]	7.5	7.4	−1.3	6.2
PAH[a]	15.0	15.1	0.7	3.4
PAH[a]	35.0	35.6	1.7	3.1
n-Acetyl-PAH[a]	7.5	7.5	0.1	1.9
n-Acetyl-PAH[a]	15.0	15.0	−0.1	1.2
n-Acetyl-PAH[a]	30.0	29.7	−1.0	2.5
Iohexol[a]	15.0	14.8	−1.3	3.5
Iohexol[a]	25.0	25.0	0.1	2.2
Iohexol[a]	45.0	44.3	−1.6	2.9
Iothalamate[b]	15.0	14.5	−3.3	4.4
Iothalamate[b]	25.0	24.7	−1.2	3.6
Iothalamate[b]	45.0	44.5	−1.1	3.1

[a] $n = 85$.
[b] $n = 10$.

sus measured component concentrations. The intra-day and inter-day precision of the method was reported as percent relative standard deviation. The method demonstrated good accuracy and precision for both plasma and urine samples with the accuracy of all components <8% and the precision within 14%. Absolute recovery for the plasma method was evaluated by comparing extracted fortified controls prepared in blank plasma versus unextracted fortified controls prepared in deionized water. The absolute recovery for the plasma method was determined to be 100% for all four components. Absolute recovery was not evaluated for urine samples as the sample preparation did not consist of an extraction (e.g. dilute urine sample with deionized water and subsequent injection). In addition, the standards and controls used for analysis were treated identical to the subject samples, thus controlling for potential errors in sample handling and micropipetting.

3.4. Chromatography

The method demonstrated excellent chromatographic selectivity with no endogenous interference at the retention times of PAH (∼4.8 min), iothalamate (∼10.2 min), iohexol (∼10.6 min) and *n*-acetyl-PAH (∼13.6 min). Chromatograms of prepared blank human plasma containing zero, low and high standard concentrations of each component (Fig. 2A–C, respectively) indicated good detector response and baseline resolution from endogenous plasma substances with an analytical run time of 23 min (allows mobile phase gradient equilibration). Chromatograms of prepared blank human urine containing zero, low and high standard concentrations of each component (Fig. 3A–C, respectively) indicated good detector response and baseline resolution from endogenous urine substances. To extend column lifetime, the analytical column was flushed after each analytical run (∼75 samples) for 1 h at 1 ml/min with acetonitrile:deionized water (90:10, v/v) to eliminate retained non-polar substances from the column.

3.5. ERPF and ERPF/GFR ratio using HPLC–UV and colorimetric analysis

For this evaluation, plasma and urine samples ($n = 6$ subjects) were evaluated using HPLC–UV and colorimetric methods of analysis for PAH and iohexol. The objective was to compare ERPF results obtained from the two analytical methods and compute the ERPF/GFR ratio, which is used for clinical impression of the subject's kidney function to the administered treatment. Since humans exhibit metabolic acetylation of PAH [5], the authors wanted to determine if there were differences in ERPF results using the two different methods of PAH analysis. It is known that the colorimetric method works by reaction of the free amino group on the aromatic moiety with the coloring agent (1% w/v, *p*-dimethylaminobenzaldehyde in 60:40 ethanol:deionized water, v/v). Since human metabolism (e.g. phase II acetylation may be weak or strong) of PAH is accomplished by reacting with the free amino group on the aromatic ring,

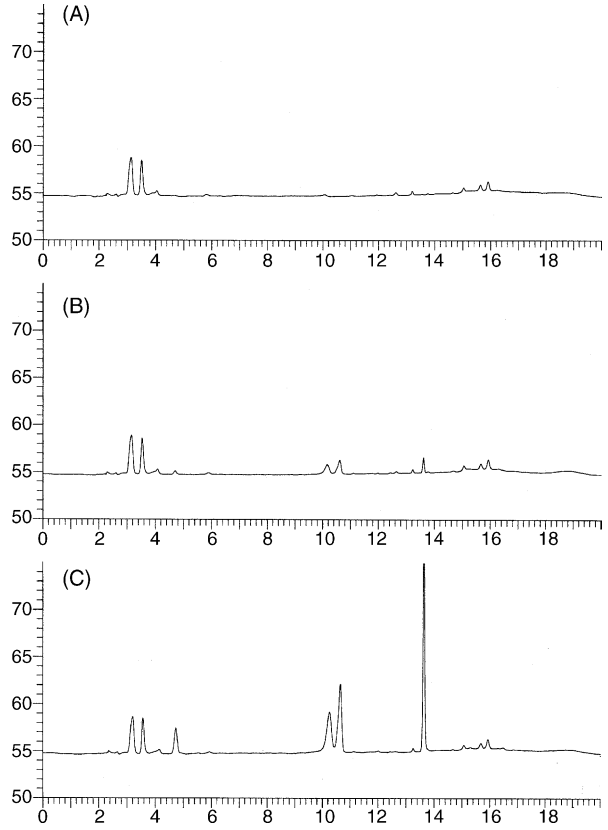

Fig. 2. Chromatograms of (A) prepared blank human plasma; (B) prepared low standard in blank human plasma fortified with 5 μg/ml PAH, 10 μg/ml iohexol, 10 μg/ml iothalamate and 2.5 μg/ml *n*-acetyl-PAH; (C) prepared high standard in blank human plasma fortified with 40 μg/ml PAH, 50 μg/ml iohexol, 50 μg/ml iothalamate and 40 μg/ml *n*-acetyl-PAH. The retention time of PAH (4.8 min), iothalamate (10.2 min), iohexol (10.6 min) and *n*-acetyl-PAH (13.6 min).

the acetylated metabolite (*n*-acetyl-PAH) will not provide a response to the coloring agent, thus lowering the total amount of PAH reported cleared by the subject using the colorimetric analysis.

ERPF is defined as the concentration of PAH [mg/ml] present in the urine sample divided by the concentration of PAH (mg/ml) present in the plasma sample times the urine volume (ml) and divided by the sample time (min) period; the resulting unit for ERPF is ml/min. As seen in Fig. 4, the colorimetric results for ERPF were higher than the HPLC results for ERPF in one subject's samples which is best explained by endogenous interferences (e.g. urea) present in the subject's urine samples which react with the color reagent causing false positive ERPF results. This result supported published literature; however with many of our subjects, the colorimetric results for ERPF were biased lower than the HPLC results for ERPF. GFR which is assessed by means of renal clearance of iohexol or iothalamte is determined by the concentration of iohexol or iothalamate (mg/ml) present in the urine sample divided by the concentration of iohexol or iothalamate (mg/ml) present in the plasma sample times the urine volume (ml) and divided by the sample time (min) period; the result-

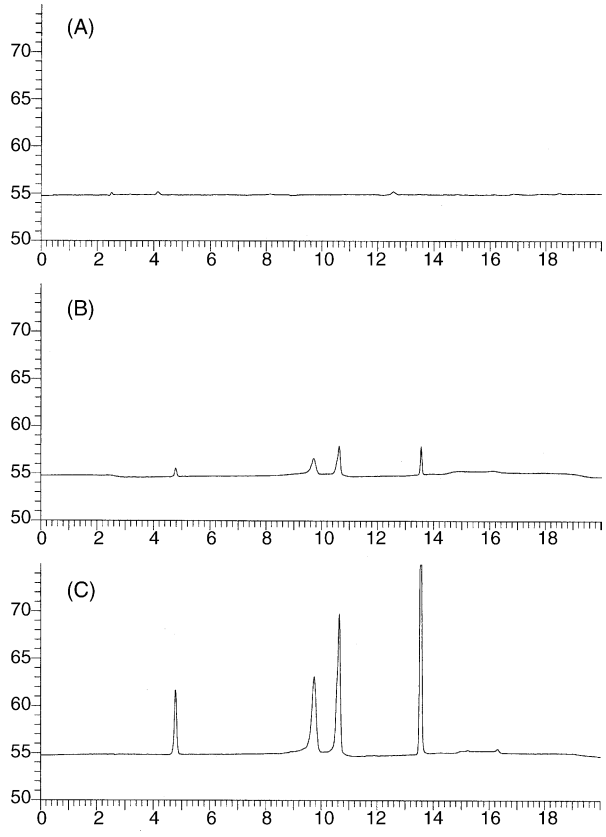

Fig. 3. Chromatograms of (A) prepared blank human urine; (B) prepared low standard in blank human urine fortified with 5 μg/ml PAH, 10 μg/ml iohexol, 10 μg/ml iothalamate and 2.5 μg/ml *n*-acetyl-PAH; (C) prepared high standard in blank human urine fortified with 40 μg/ml PAH, 50 μg/ml iohexol, 50 μg/ml iothalamate and 40 μg/ml *n*-acetyl-PAH. The retention time of PAH (4.8 min), iothalamate (9.7 min), iohexol (10.6 min) and *n*-acetyl-PAH (13.6 min).

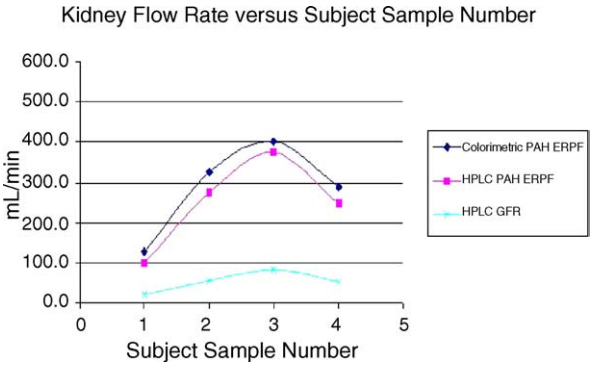

Fig. 4. Plot of ERPF and GRF results using colorimetric and HPLC analysis. Data represents one subject's results from one treatment day.

ing unit for GFR is ml/min. Since the typical value reported for the ERPF/GFR ratio is approximately 5 (or 0.2 if the ratio is defined as GFR/ERPF), the authors computed each subject's ERPF/GFR ratio to help determine which method provided an ERPF/GFR ratio closer to the expected value (Table 3).

The HPLC–UV method separated and quantified both PAH and the *n*-acetyl-PAH metabolite making it possible to estimate the total clearance of PAH (PAH plus *n*-acetyl-PAH). However, it was found that with all our subjects, the *n*-acetyl-PAH was not cleared from the body as quickly as the PAH itself. The last urine sample representing 12–24 h clearance obtained from each subject still had significant levels of *n*-acetyl-PAH present with no detectable levels of PAH present. Intuitively this makes sense, as the acetylated PAH is less polar than PAH due to addition of the non-polar acetyl group to the polar PAH moiety; thus making it less polar and increasing its biological half-life (longer clearance). This phenomenon made calculating ERPF from combining the HPLC PAH and *n*-acetyl-PAH results inaccurate as the accumulation of *n*-acetyl-PAH in the urine caused the ERPF results to be biased higher (up to 15%) in the subject's subsequent urine samples due to increasing its biological half-life (due to slower clearance). Therefore the metabolite *n*-acetyl-PAH should not be used in ERPF computations from HPLC analysis. The only benefit we found for quantifying the metabolite was to help determine the subject's percent acetylation of PAH which ranged from 15–18% and are listed in Table 3. Subjects from our other clinical studies had PAH acetylation mean values ($n = 15$ samples per subject) ranging from 8 to 35% (grand mean 17%, $n = 62$ subjects, Fig. 5), which supports the notion of an individual being genetically predisposed (polymorphism) to being a weak or strong acetylator via metabolism.

Table 3 lists the results of the six subjects ERPF/GFR ratios using HPLC–UV and colorimetric analysis. For this study, each subject was administered three treatments as per institution approved study protocol with four samples each of plasma and urine collected and evaluated to calculate ERPF and GFR. As can be seen from the table, the HPLC ERPF/GRF ratios for these subjects were very close to the

Table 3
Comparison of colorimetric and HPLC ERPF/GRF ratios

Subject	Treatment days, samples per treatment	Mean colorimetric ERPF/GFR ratio	Mean HPLC ERPF/GFR ratio	%Mean PAH acetylation
1	3, 4	7.6	6.2	15
2	3, 4	2.7	4.8	18
3	3, 4	2.2	5.4	15
4	3, 4	2.0	5.5	15
5	3, 4	1.7	4.5	16
6	3, 4	5.0	5.0	15

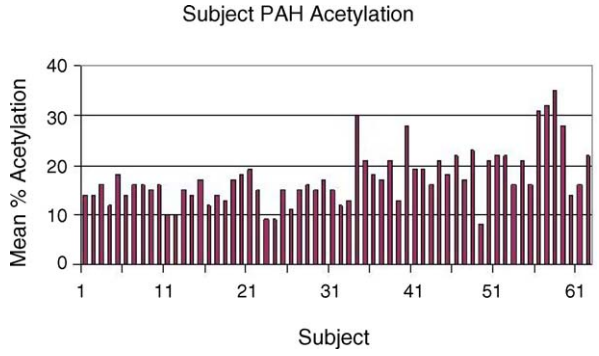

Fig. 5. Bar chart of 62 study subject's percent mean PAH acetylation values.

predicted value of 5. However, several of the colorimetric ERPF/GRF ratios were less than the predicted value of 5. Closer examination of the raw data for these subjects found that the colorimetric values for PAH in the plasma samples were biased higher than those obtained using HPLC analysis. This positive bias would cause the colorimetric ERPF result to be lower as the plasma PAH result is in the denominator of the calculation for ERPF; thus causing a lower ERPF result and subsequent low ERPF/GRF ratio. It is unknown to the authors at this time what would cause this phenomenon in the colorimetric analysis of these subjects's plasma samples.

4. Conclusions

A sensitive and selective method has been developed for evaluating PAH, *n*-acetyl-PAH, iohexol and iothalamate in plasma and urine. The method employed a simple sample preparation for plasma and urine samples and eliminated the need for an internal standard and ion-pairing reagent. In addition, this method utilized current bioanalytical HPLC column technology, which provided sufficient selectivity and sensitivity for measurement of these four components simultaneously to calculate subject kidney GFR, ERPF and ERPF/GFR ratio. The method was employed without significant methodological problems in the evaluation of several thousand plasma and urine samples obtained from clinical pharmacodynamic studies.

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