Journal of Chromatography, 414 (1987) 275–284
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3444

DETERMINATION OF FREE AND TOTAL PHENYLACETIC AND *p*- AND *m*-HYDROXYPHENYLACETIC ACIDS IN HUMAN URINE BY LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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(First received June 18th, 1986; revised manuscript received October 13th, 1986)

SUMMARY

A highly sensitive and rapid liquid chromatographic method for the determination of free and total phenylacetic and *p*- and *m*-hydroxyphenylacetic acids in human urine is described. After extraction of urine with diethyl ether, these acids and phenylpropionic acid (internal standard) are converted into the corresponding fluorescent derivatives by treatment with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone in the presence of potassium hydrogen carbonate and 18-crown-6 in acetonitrile. The derivatives are separated on a reversed-phase column (Radial-Pak cartridge C_{18}) with aqueous 65% (v/v) methanol and detected fluorimetrically. The detection limits for phenylacetic and *p*- and *m*-hydroxyphenylacetic acids are 5, 30 and 100 fmol, respectively, at a signal-to-noise ratio of 5 in a 20-μl injection volume. This sensitivity permits precise determination of the free and total acids in 20 μl of normal human urine.

INTRODUCTION

Phenylacetic (PAA) and *p*- and *m*-hydroxyphenylacetic acids (p- and m-HPAs, respectively) are excreted as free and conjugated forms in human urine. PAA may be mainly derived from phenylalanine either by decarboxylation to 2-phenylethylamine and further deamination by monoamine oxidase to PAA, or by transamination to phenylpyruvic acid followed by decarboxylation to PAA. p- and m-HPAs may also be derived from *p*- and *m*-tyrosines, respectively, through the same pathway. Correlation between depressive [1], chronic schizophrenic [2] and phenylketonuric disorders [3–5] and the levels of urinary PAA and HPAs (both free and conjugated forms) have been reported. It has been indicated that the abnormal amounts of the acids are observed in patients with the described disorders. Since the changes, however, were not large in many cases, conclusive

0378-4347/87/$03.50

results have not been obtained. This may be partially due to the lack of a sensitive, simple and reproducible method.

Various methods, including spectrophotometric [6], gas chromatographic (GC) [7–12] and high-performance liquid chromatographic (HPLC) [13] methods, have been used for the determination of free and/or total (the sum of free and conjugated) PAA and/or HPAs in human urine. However, these methods are not sensitive enough to determine the urinary acids simultaneously in an extremely small amount of sample. GC–mass spectrometric (GC–MS) methods have been developed for the determination of urinary PAA and/or HPAs [9,14]. Although these methods are very sensitive, they require expensive equipment and rather tedious technique. Thus, they have not been used routinely.

We have developed 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ) as a highly sensitive fluorescence derivatization reagent for carboxylic acids [15,16]. The purpose of the present research is to establish a highly sensitive, simple and reproducible HPLC method using Br-DMEQ for the simultaneous determination of PAA and HPAs in a minute amount of human urine. Phenylpropionic acid (PPA), which is not present in human physiological fluids, is used as an internal standard (I.S.).

EXPERIMENTAL

Reagents and chemicals

All chemicals and solvents were of analytical-reagent grade, unless stated otherwise. Deionized and distilled water was used. PAA, p- and m-HPAs and PPA were purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile used for the derivatization reaction was purified as described previously [16]. Br-DMEQ was prepared as described previously [16]. Br-DMEQ (1.3 m*M*), 18-crown-6 (3.8 m*M*) and PPA (32.0 μM, I.S.) solutions were prepared in acetonitrile. The Br-DMEQ solution could be used for more than 1 week when stored in a refrigerator at 4°C.

Apparatus

Uncorrected fluorescence spectra and intensities were measured with a Hitachi 650-60 spectrofluorimeter (Tokyo, Japan) in 10×10 mm quartz cells; spectral bandwidths of 10 nm were used both for the excitation and emission monochromators.

Infrared (IR) spectra were recorded with a Shimadzu 430 IR spectrophotometer (Kyoto, Japan) in potassium bromide pellets. ^{1}H Nuclear magnetic resonance (NMR) spectra were obtained with a Hitachi R-90H spectrometer at 90 MHz using ca. 5% (w/v) solution in [^{2}H]chloroform containing tetramethylsilane as I.S. Splitting patterns were designated as follows: s, singlet; m, multiplet. Electron-impact mass spectra were taken with a JEOL DX-300 spectrometer (Tokyo, Japan).

Radial-Pak cartridges C_{18}, C_8, phenyl and CN (all 100×8 mm I.D.; 10 μm particle size), YMC Pack C_8 (150×6 mm I.D.; 10 μm particle size) and LiChrosorb RP-8 (150×4 mm I.D.; 10 μm particle size) were purchased from Waters Assoc.

(Tokyo, Japan), Yamamura Chemical Labs. (Kyoto, Japan) and Merck (Tokyo, Japan), respectively.

A Hitachi 655A high-performance liquid chromatograph equipped with a high-pressure sample injector and a Hitachi F1000 fluorescence spectromonitor equipped with a 12-μl flow-cell operating at the excitation and emission wavelengths of 370 nm and 455 nm, respectively, were used. The column was a Radial-Pak cartridge C_{18}. The mobile phase was water–methanol (35:65, v/v). The flow-rate was 2.0 ml/min (ca. 70 kg/cm^2). The column temperature was maintained at 40 ± 1 °C. This column could be used for more than 1000 injections with only a small decrease in the theoretical plate number when washed with methanol at a flow-rate of 2 ml/min for ca. 20 min at the end of each working day. Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi 650-60 fluorescence spectrophotometer fitted with a 20-μl flow-cell; the spectral bandwidths were 5 nm in both the excitation and emission monochromators.

Fluorescent derivatives of phenylacetic acid

Br-DMEQ (100 mg, 0.32 mmol), PAA (44 mg, 0.32 mmol) and 18-crown-6 (42 mg, 0.16 mmol) were dissolved in 15 ml of acetonitrile. To the solution placed in a screw-capped 10-ml test-tube were added ca. 10 mg of anhydrous potassium hydrogen carbonate. The tube was tightly closed and heated at 50 °C for ca. 60 min and cooled. Potassium hydrogen carbonate was removed by filtration and washed two or three times with small portions of acetonitrile. The combined filtrates and washings were evaporated to dryness in vacuo. The residue, dissolved in 5 ml of *n*-hexane–ethyl acetate (3:2, v/v), was chromatographed on a silica gel 60 (ca. 75 g, 70–230 mesh; Japan Merck) column (25 × 2 cm I.D.) with the same solvent. The main fraction was evaporated to dryness and the residue was recrystallized from *n*-hexane as pale yellow needles (m.p. 154.5–156.5 °C; yield, 50 mg). Analytical data were as follows: IR ν_{max} (cm^{-1}: 1745 [O(C=O)]; 1645 (C=O); 1635 (aromatic C=N and/or C=C); ^{1}H NMR (ppm): 3.67 (3H, s, N–CH_3); 3.80 [2H, s, (C=O)CH_2]; 3.93 and 4.00 (3H each, s each, O–CH_3); 5.37 [2H, s, CH_2–O(C=O)], 6.66 and 7.12 (1H each, s each, quinoxalinyl H)], 7.31–7.42 (5H, m, phenyl H); analysis calculated for $C_{20}H_{20}N_2O_5$: C, 65.21; H, 5.47; N, 7.60; found; C, 65.11; H, 5.41; N, 7.41. Mass spectra *m/z*: 368 (M^+); 250 [M^+ – (C=O)CH_2–C_6H_5 + H, base peak].

Urine samples

Urine (24 h) from healthy volunteers was collected in the presence of 10 ml of 0.3 *M* disodium ethylenediaminetetraacetate as a preservative. The urine samples were frozen at −40 °C immediately after collection and analysed within 2 weeks.

The sample solution for the determination of total acids was prepared as follows. The urine (20 μl) was mixed with 20 μl of 6 *M* hydrochloric acid, and the mixture was hydrolysed at 100 °C for 90 min. To the resulting solution, 2 ml of diethyl ether and 50 μl of the PPA (I.S.) solution were added. The resulting mixture was vortexed for ca. 2 min and centrifuged at 1000 *g* for 2 min. The

organic layer (ca. 1.4 ml) was evaporated to dryness in vacuo at 15–20°C, and the residue was dissolved in 200 μl of acetonitrile. A 100-μl portion of the final solution was used for analysis.

For the determination of free acids, the same procedure was carried out except that hydrolysis was omitted.

Derivatization procedure

A 100-μl portion of a sample solution was placed in a screw-capped 10-ml vial, to which were added ca. 20 mg of a mixture of potassium hydrogen carbonate and potassium sulphate (1:7, w/w) and 50 μl each of the Br-DMEQ and 18-crown-6 solutions. The vial was tightly closed and warmed at 50°C for 30 min in the dark. After cooling, 20 μl of the resulting mixture were injected into the chromatograph.

The calibration graphs were prepared according to the procedures for the sample preparation and the derivatization, except that 50 μl of the PPA (I.S.) solution was replaced with the I.S. solution containing between 50 pmol and 100 nmol each of PAA and p- and m-HPAs. The net peak-height ratios of the individual acids and PPA were plotted against the concentrations of the acids spiked.

RESULTS AND DISCUSSION

HPLC conditions

The separation of the DMEQ derivatives of PAA, p- and m-HPAs and PPA was studied on reversed-phase columns, described in the Experimental section, using methanol, acetonitrile, water and their mixtures as mobile phases. The best separation was achieved using a Radial-Pak cartridge C_{18} and water–methanol (35:65, v/v) as the eluent. Fig. 1 shows a typical chromatogram obtained with a mixture of these acids. The peaks for PAA, p- and m-HPAs and PPA (retention times, see Table I) could be completely separated from the components of the reagent blank (Fig. 1, peaks 6 and 7) within 26 min. The individual acids gave single peaks in the chromatogram. When the column temperature was maintained at 40 ± 1°C, reproducible retention times were obtained for the four acids tested under the HPLC conditions described.

Derivatization conditions

Br-DMEQ gave the most intense peaks for the four acids examined at concentrations greater than ca. 1.0 m*M* in the solution; thus the concentration of 1.3 m*M* was chosen. Maximum and constant peak heights were attained at 18-crown-6 concentration in the solution in a range 1.9–5.7 m*M*; 3.8 m*M* was selected for the procedure. The peak heights were maximal and constant when the amount of potassium hydrogen carbonate was 2–30 mg; peak 7 (Fig. 1), due to the reagent blank, increased in height and width with increasing amount of carbonate and this interfered with the determination of PAA and m-HPA. Thus, 2.5 mg of potassium hydrogen carbonate was used in the procedure. In order to obtain sufficient reproducibility, a mixture of potassium hydrogen carbonate and potassium sulphate (1:7, w/w) was used; the sulphate did not interfere with the determination

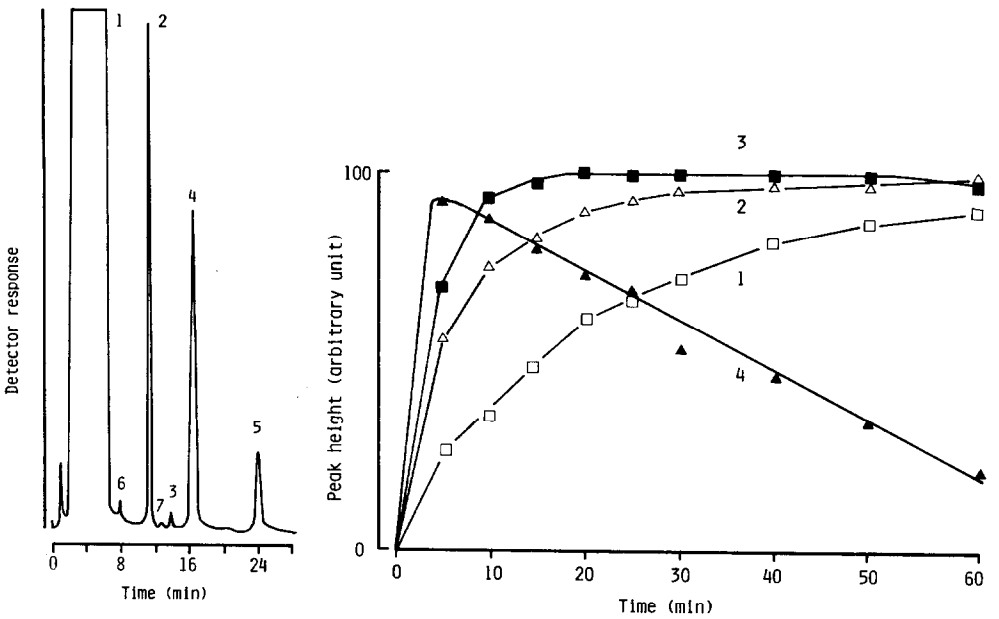

Fig. 1. Chromatogram of the DMEQ derivatives of PAA, m- and p-HPAs and PPA. An aliquot (100 μl) of a standard mixture of the acids (10 nmol each per ml) in acetonitrile was treated according to the derivatization procedure. Peaks: 1 = Br-DMEQ; 2 = PAA; 3 = m-HPA; 4 = PPA; 5 = p-HPA; 6 and 7 = the reagent blank.

Fig. 2. Effect of the reaction time on the fluorescence derivatization of PAA. Aliquots (100 μl) of PAA (1.0 nmol/ml) were treated as in the derivatization procedure at various temperatures. Temperatures: (1) 20°C; (2) 37°C; (3) 50°C; (4) 80°C.

TABLE I

RETENTION TIMES AND RELATIVE PEAK HEIGHTS OF THE DMEQ DERIVATIVES OF BIOGENIC AROMATIC ACIDS AND PPA

Acids	Relative peak height★	Retention time (min)
PAA	100	11.2
m-HPA	4	13.8
PPA	63	16.4
p-HPA	16	24.0
Benzoic acid	40	5.2
p-Hydroxybenzoic acid	1	5.0
Mandelic acid	2	7.8
p-Hydroxymandelic acid	1	5.6
Phenyllactic acid	1	6.1
p-Hydroxyphenyllactic acid	1	5.0
Vanylmandelic acid	3	20.3
Homovanillic acid	2	28.2

★The peak height for PAA was taken as 100.

of the acids. When potassium carbonate was used, peak 7 became large and broad compared with that obtained with the potassium hydrogen carbonate.

The derivatization reaction of the four acids with Br-DMEQ, which apparently occurred even at moderately low temperatures, was accelerated by higher temperatures. An example for PAA is shown in Fig. 2. However, at 80°C, the peak heights for all the compounds decreased with heating time. At 50°C, the peak heights reached a maximum after warming for 20 min, but peak 7 (Fig. 1) increased in height with prolonged heating time. Thus, 30-min warming at 50°C was recommended in the procedure. The DMEQ derivatives of all the acids examined were stable for at least 2 h in daylight and for at least 72 h in the dark at room temperature.

The detection limits for PAA, p- and m-HPAs and PPA were 5, 30, 100 and 8 fmol, respectively, in a 20-μl injection volume at a signal-to-noise ratio of 5.

Fluorescent derivative in the determination of PAA

In order to investigate the structure of the fluorescent products, PAA was employed as a model compound. The reaction product from PAA was identified as 6,7-dimethoxy-1-methyl-3-benzoyloxymethyl-2(1*H*)-quinoxalinone by the analytical data described under Experimental. The fluorescence excitation (maximum, 370 nm) and emission (maximum, 455 nm) spectra of the product in aqueous 65% (v/v) methanol were almost identical with those of the eluates for the other acids, respectively. These results indicate that the fluorescent products of the acids might be the corresponding esters.

Determination of PAA, and p- and m-HPAs in urine

Hydrolysis. The optimal conditions for hydrolysis of urinary conjugated PAA and HPAs were examined by using a sample of normal human urine. When the urine was acidified with an equal volume of 6 *M* hydrochloric acid, all the conjugated acids tested were almost completely hydrolysed at 100°C for 60–120 min, as shown in Fig. 3. Thus, the acidified urine was heated at 100°C for 90 min in the procedure for the determination of the total acids.

Extraction. PAA and HPAs were effectively extracted from the acidified urines before and after hydrolysis with diethyl ether. Recovery tests were performed by adding known amounts (5.0 nmol each) of the acids to the acidified urines (100 μl). Individual recoveries (%, mean $\pm$S.D., $n=10$) of PAA, p- and m-HPAs and PPA were 93.2 ± 2.8, 90.1 ± 4.8 and 91.2 ± 4.0, respectively. When benzene or chloroform was used, only PAA was extracted. Less satisfactory recoveries were found with ethyl acetate.

Chromatography. Fig. 4A and B shows typical chromatograms obtained with pooled human urines before and after hydrolysis. The components of peaks 2, 3 and 4 (Fig. 4) were identified as the DMEQ derivatives of PAA and m- and p-HPAs, respectively, on the basis of their retention times and the fluorescence excitation (maximum, 370 nm for all the compounds) and emission (maximum, 455 nm for all the compounds) spectra of the eluates of the peaks by comparison with those in Fig. 1, and also by co-chromatography. Some unidentified peaks (peaks 8 and 9, Fig. 4) were observed in the chromatograms from urine. These

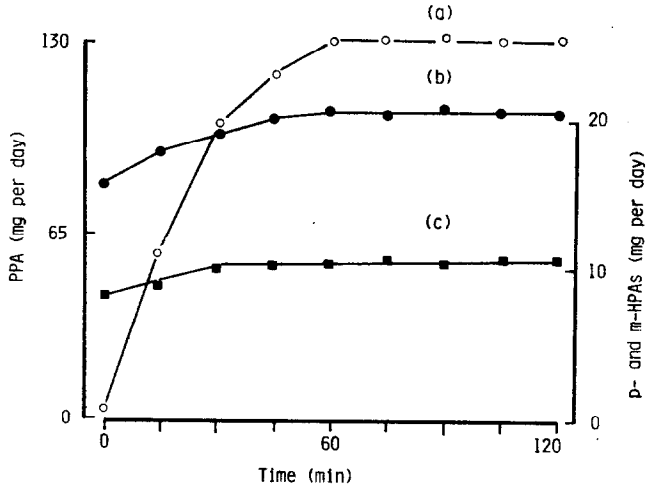

Fig. 3. Effect of the reaction time on the hydrolysis of conjugated (a) PAA, (b) p-HPA and (c) m-HPA. An aliquot (20 μl) of the urine was treated as in the procedure.

peaks increased in height in proportion to urine sample size. In addition, each eluate from peaks 8 and 9 exhibited fluorescence excitation and emission maxima around 370 and 455 nm, almost identical with those of peaks 2–5. These obser-

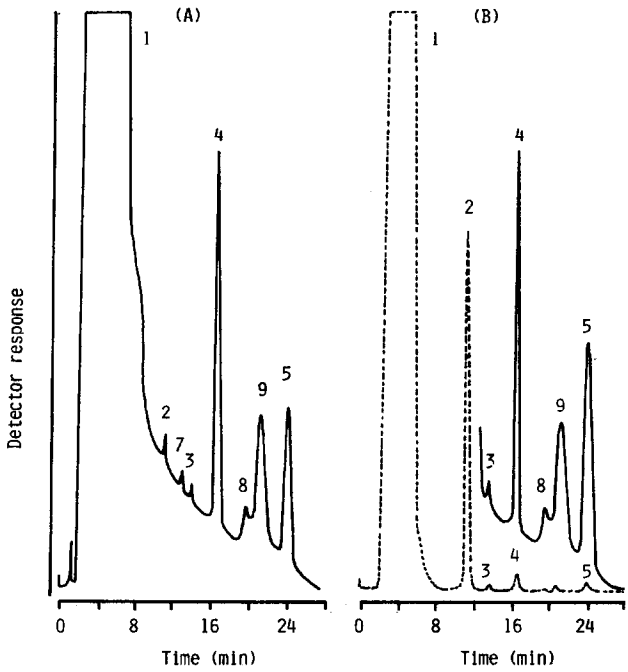

Fig. 4. Chromatograms of the DMEQ derivatives of PAA, p- and m-HPAs in human urines (A) before and (B) after hydrolysis. Experimental details are described in the procedure. For peaks 1–7, see Fig. 1; peaks 8 and 9 are unidentified. Detector sensitivity: solid line = 2; dashed line = 0.1.

vations suggest that peaks 8 and 9 may be due to unknown endogenous carboxylic acids in urine. However, they did not interfere with the determination of PAA and HPAs in urine. Thus, further clean-up of the sample solution was not necessary.

Interferences. Many biogenic carboxylic acids that can be extracted from urine did not give fluorescent derivatives [16]. Some aromatic acids, commonly present in human urine, also reacted with Br-DMEQ to produce fluorescent derivatives (Table I). However, their derivatives eluted at different retention times from those of PAA, HPAs and PPA, and so did not interfere with the determination of the acids. The DMEQ derivatives of biogenic carboxylic acids, such as dicarboxylic (oxalic, malonic, succinic and adipic acids), hydroxycarboxylic (lactic and malic acids), imidazole- and N-methylimidazoleacetic acids, indole- and 5-hydroxyindoleacetic acids, were co-eluted with Br-DMEQ under the HPLC conditions used. On the other hand, the derivatives of long-chain, saturated and unsaturated fatty (C_{12}–C_{22}) acids were strongly retained on the column and were not eluted. Thus, these compounds did not interfere with the determination of PAA and HPAs even when they were spiked at unusually high concentrations in human urine (10.0 nmol per 20 μl each). However, since the DMEQ derivatives of the fatty acids in the urine may slowly alter the retention properties of the column, the column was washed with methanol every day after analyses to remove the derivatives.

Linearity and precision. Linear relationships were observed between the ratios of the peak heights of PAA and HPAs to that of PPA and the amounts of PAA and HPAs (50 pmol to 100 nmol) added to 40 μl of the acidified urine (corresponding to ca. 0.88 pmol to 1.76 nmol per injection volume). The linear regressions (the linear correlation coefficients in parentheses) of the curves were $y=0.02051x+0.0021$ ($r=0.998$) for PAA, $y=0.00312x+0.0010$ ($r=0.995$) for p-HPA and $y=0.00078x+0.0008$ ($r=0.996$) for m-HPA; where y and x are the peak-height ratio and the concentration (nmol/ml) of the individual acids, respectively.

The within-day precision was determined from repeated analyses ($n=20$) of a normal human urine containing 11.7, 162.1 and 55.0 nmol/ml of free PAA, and p- and m-HPAs, and 394, 301 and 63 nmol/ml of total PAA, and p- and m-HPA, respectively. The coefficients of variation (C.V.s) did not exceed 4% for both free and total acids. The between-day precision was obtained by performing the analyses ($n=3$ each day) using the calibration graphs prepared on that day during 10 days with the same sample kept frozen at $-40°C$. The C.V.s were 3.2, 4.5 and 5.3% for free PAA, and p- and m-HPAs, and 4.4, 4.5 and 3.9% for total PAA and p- and m-HPAs, respectively.

Free and total PAA and HPAs in human urine. The levels of free and total PAA and HPAs in human urines are given in Table II. The mean values for total PAA were in good agreement with those obtained by other workers [1,7,8,10–13]. For free PAA, our value agreed closely with those listed by Davis and Boulton [9] and Goodwin et al. [10], although it was lower than that obtained by Martin et al. [14]. The mean values of free and total HPAs obtained in this study were similar to those reported by Sandler et al. [12] and Davis et al. [9].

TABLE II

URINARY EXCRETION (24 h) OF PAA, AND p- AND m-HPAs IN URINES FROM HEALTHY VOLUNTEERS

Values are in mg (μmol) per day.

Age (years)	Sex★	PAA		p-HPA		m-HPA	
		Free	Total	Free	Total	Free	Total
38	M	0.48 (3.4)	233.8 (1670)	38.7 (258)	60.4 (402)	10.4 (69)	12.2 (81)
35	M	1.64 (11.7)	55.1 (394)	24.3 (162)	45.1 (301)	8.2 (55)	9.5 (63)
33	M	1.38 (9.9)	150.8 (1077)	18.5 (123)	24.3 (162)	6.5 (43)	8.5 (57)
28	M	0.86 (6.1)	138.5 (989)	20.5 (137)	25.3 (175)	10.2 (68)	13.3 (89)
27	M	0.70 (5.0)	125.3 (895)	22.8 (152)	25.8 (172)	5.8 (39)	6.5 (43)
26	M	0.81 (5.8)	140.2 (1000)	29.5 (197)	37.3 (245)	5.5 (37)	6.5 (43)
25	M	0.73 (5.2)	112.5 (804)	18.5 (123)	23.2 (155)	4.8 (32)	5.8 (39)
24	M	0.28 (2.0)	136.8 (977)	16.1 (107)	17.9 (119)	6.9 (46)	8.2 (55)
22	M	0.14 (1.0)	233.3 (1607)	19.0 (127)	35.2 (235)	7.8 (52)	9.5 (63)
21	M	0.39 (2.8)	120.3 (859)	15.8 (101)	23.2 (155)	9.3 (62)	12.3 (82)
33	F	0.25 (1.8)	110.5 (789)	16.5 (110)	20.2 (135)	13.8 (92)	15.5 (103)
22	F	1.07 (7.9)	73.1 (522)	7.2 (48)	8.4 (56)	10.5 (70)	12.3 (82)
21	F	0.97 (7.1)	84.4 (603)	17.1 (114)	17.5 (117)	4.5 (30)	7.1 (47)
21	F	0.68 (5.0)	61.5 (439)	16.9 (113)	19.1 (127)	4.8 (32)	8.1 (54)
21	F	1.46 (10.7)	176.2 (1259)	16.6 (111)	38.8 (259)	5.5 (37)	10.2 (68)
Mean		0.79 (5.7)	130.8 (934)	19.9 (133)	28.1 (187)	7.6 (51)	9.7 (65)
S.D.		0.44 (3.2)	51.5 (368)	6.9 (46)	12.6 (84)	2.6 (17)	2.8 (19)

★M = male; F = female.

CONCLUSION

The described assay is the first HPLC method with fluorimetric detection for the simultaneous determination of PAA and HPAs in human urine. The method has adequate sensitivity to measure the acids in 20 μl of normal urine. The method is readily performed and should therefore be useful for physiological and pharmacological investigations of these acids.

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