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Determination of Free and Total Phenylacetic Acid in Human and Rat Plasma by High-Performance Liquid Chromatography with Fluorescence Detection

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A highly sensitive and simple high-performance liquid chromatographic method has been developed for the determination of free and total phenylacetic acid in human and rat plasma. After extraction with diethyl ether from plasma, phenylacetic acid and phenylpropionic acid (internal standard) are converted to the corresponding fluorescent derivatives by reaction 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 limit for phenylacetic acid is 11 pmol/ml in plasma at a signal-to-noise ratio of 5. This sensitivity permits precise determination of free and total phenylacetic acid in 50 μl of human and rat plasma. The method was applied to the determination of free and total phenylacetic acid in plasma from healthy volunteers, and control and "behavioral despair" rats.

Keywords——phenylacetic acid; human plasma; rat plasma; high-performance liquid chromatography; fluorescence detection; 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone

Phenylacetic acid (PAA) is present as free and conjugated forms in human plasma. PAA may be mainly derived from phenylalanine and phenylethylamine by decarboxylation and deamination, and is further metabolized in the human body to its glutamine conjugate. It is indicated that the amount of PAA decreases in plasma of patients with depressive illness.[1,2] Therefore, the determination of plasma PAA in humans may be useful for the diagnosis, monitoring and investigation of depressive illness.

Recently, rats forced to swim in a restricted space have been found to be a specific animal model for depressive illness. Thus, the determination of plasma PAA in such rats may improve our understanding of depressive illness.[3]

Gas chromatography-mass spectrometric (GC-MS) methods have been most widely used for the determination of free and total (the sum of free and conjugated) PAA in human plasma.[2,4–7] Although the methods are very sensitive, they require expensive equipment and rather tedious techniques. Thus, they have not been routinely used. Recently, a simple high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection has been proposed for the determination of total PAA in human plasma.[1] However, the method has a limited sensitivity and thus requires a large amount of human plasma (2 ml). Furthermore, the method has not been applied to the determination of free PAA, which occurs in a minute amount in human plasma. No method has yet been applied to experimental small animals such as rats and mice.

We previously reported a simple and sensitive HPLC method for the simultaneous determination of free and total PAA and *p*- and *m*-hydroxyphenylacetic acids in human urine using precolumn fluorescence derivatization with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone (Br-DMEQ), a fluorogenic reagent for carboxylic acids; these acids,

after extraction with diethyl ether from urine, are converted into the corresponding fluorescent compounds by reaction with Br-DMEQ and these compounds are separated on a reversed-phase (Radial Pak cartridge C_{18}) column with isocratic elution.[8] The purpose of the present research was to establish a simple, sensitive and rapid method for the determination of free and total PAA in a minute amount of human and rat plasma. The established method was used to compare free and total PAA concentrations in control rat plasma with those in plasma from rats forced to swim. Phenylpropionic acid (PPA), which is not present in human and rat physiological fluids, was used as an internal standard (IS).

Experimental

Chemicals and Solutions——All chemicals and solvents were of reagent grade, unless otherwise stated. Deionized and distilled water was used. PAA was purchased from Sigma (St. Louis, Mo., U.S.A.). Acetonitrile used for the derivatization reaction was purified as described previously.[9] Br-DMEQ was prepared as described previously;[9] it is now available from Dojindo Laboratories (Kumamoto, Japan). Br-DMEQ (1.3 mM), 18-crown-6 (3.8 mM) and PPA (3.2 μM, IS) solutions were prepared in acetonitrile. The Br-DMEQ solution could be kept for more than one week when stored in a refrigerator at 4 °C.

Apparatus and HPLC Conditions——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 379 and 455 nm, respectively, were used. The column was a Radial Pak cartridge C_{18} (100 × 8 mm i.d.; particle size, 5μm; Waters Assoc., Milford, Mass., U.S.A.). The mobile phase was H_2O–MeOH (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.

Plasma Samples——Male Wister rats ($n = 20$) weighing 210—230 g were used for the present study. The rats were housed in a well-controlled environment with free access to food and water, and were used within a day after being brought into the laboratory. The rats were divided into two groups of 10 rats each. One group (intact rats) served as a control. The other group (10 rats) was treated according to the novel forced swimming test of Porsolt *et al.*[3] Briefly, the rats were individually forced to swim once daily inside plexiglass cylinders containing water maintained at 25 °C for 15 min. After 4 daily sessions of swimming, the total duration of immobility was measured during a 5 min test. All the rats employed were judged from the forced swimming test to be in a depressive state. On the next day, the rats were killed by decapitation, and blood (2—4 ml) was collected in a centrifuge tude containing disodium ethylenediaminetetraacetate (2—4 mg) as an anticoagulant. Plasma was separated by centrifugation of the blood at 10000 ***g*** at 5 °C. Human plasma was obtained from fasting healthy volunteers in our laboratory. Human and rat plasma samples were stored at −40 °C until just before use.

Procedure——A 50-μl portion of plasma sample was diluted with 50 μl of the PPA (IS) solution, 100 μl of 0.2 M zinc sulfate, and 100 μl of 0.2 M barium hydroxide. The mixture was centrifuged at 6500 ***g*** for 20 min. The supernatant (deproteinized plasma; 200 μl) was mixed with 50 μl of 6 M hydrochloric acid, and the acidified plasma was hydrolyzed at 100 °C for 90 min. To the resulting solution, 2 ml of diethyl ether was added, and 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 placed in a screw-capped 10-ml vial, to which were added *ca.* 20 mg of a mixture of potassium hydrogen carbonate and potassium sulfate (1 : 4, 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 was injected into the chromatograph. For the determination of free PAA, the same procedure was carried out except that hydrolysis was omitted.

The calibration graphs were prepared according to the standard procedure except that 50 μl of the PPA (IS) solution was replaced with the IS solution containing 50 pmol—100 nmol of PAA. The net peak height ratio of PAA was plotted against the concentration of PAA spiked.

Results and Discussion

HPLC and derivatization conditions were the same as described previously.[8]

Deproteinization

Plasma had to be deproteinized, otherwise the HPLC column packing was considerably

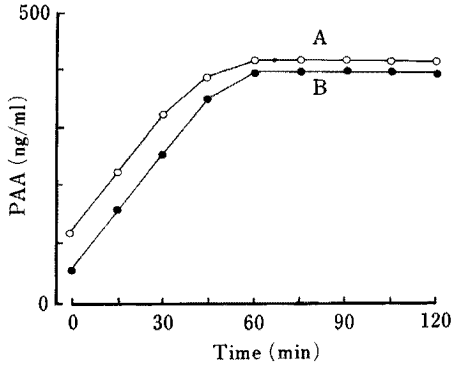

Fig. 1. Effect of the Reaction Time on the Hydrolysis of Conjugated PAA in (A) Human and (B) Control Rat Plasma

Portions (50 μl) of the plasma were treated according to the standard procedure.

damaged. The deproteinization was effectively done by adding zinc sulfate and barium hydroxide to plasma. When plasma was deproteinized with perchloric acid, an unknown, broad and large peak appeared at the retention time of 10—30 min on the chromatogram.

Hydrolysis

The optimal conditions for hydrolysis of conjugated PAA were examined by using pooled human and rat plasma. When the deproteinized plasma was acidified with an equal volume of 6 M hydrochloric acid, and hydrolyzed at 100 °C for 60—120 min, the conjugated PAA in human and rat plasma was almost completely hydrolyzed, as shown in Fig. 1. Thus, the acidified plasma was heated at 100 °C for 90 min in the procedure for the determination of total PAA.

Extraction

PAA was effectively extracted from the acidified plasma before and after hydrolysis with diethyl ether. A recovery test was performed by adding a known amount (50 pmol) of PAA to human plasma (50 μl). Recovery of PAA was $50.2 \pm 3.2\%$ (mean $\pm$ S.D., $n = 10$). Main loss in PAA occurred in the protein precipitation step. Less satisfactory recoveries were found with ethyl acetate, benzene and chloroform. Similar results were also obtained for rat plasma.

Chromatography

Figure 2 shows a typical chromatogram obtained with a standard mixture of PAA, *p*- and *m*-hydroxyphenylacetic acids and PPA.[8] The peaks for the acids (peaks 2—5) could be completely separated from the components of the reagent blank (Fig. 2, peaks 6 and 7) within 26 min. Figures 3A and B show typical chromatograms obtained with pooled human and rat plasma, respectively, before and after hydrolysis. The component of peak 2 was identified as the DMEQ derivative of PAA on the basis of the retention time and the fluorescence excitation (maximum, 370 nm) and emission (maximum, 455 nm) spectra of the peak fraction by comparison with those in Fig. 2, and also by co-chromatography of the standard compound and plasma with aqueous 50—100% methanol as the mobile phase. On the other hand, no peaks for *p*- and *m*-hydroxyphenylacetic acids were observed in the chromatograms, because the acids occur in extremely small amounts in human plasma.[2,5,7] Some unidentified peaks (Fig. 3A and B, peaks 8—10) were observed on the chromatogram. The heights of these peaks increased in proportion to the plasma sample size. In addition, each eluate from peaks 8—10 exhibited fluorescence excitation and emission maxima around 370 and 455 nm, almost identical with those of peaks 2—5 (Fig. 2). These observations suggest that peaks 8—10 may be due to unknown endogeneous carboxylic acids in plasma. However, they did not interfere with the determination of PAA in plasma. No conversions of phenylacetaldehyde and

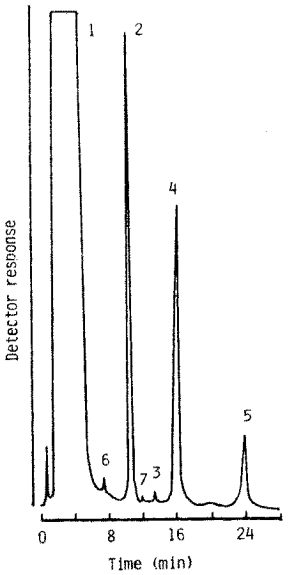

Fig. 2. Chromatogram of the DMEQ Derivatives of PAA, *p*- and *m*-Hydroxyphenylacetic Acids and PPA

A portion (50 μl) of a standard mixture of the acids (10 nmol each/ml) in water was treated according to the standard procedure. Peaks: 1, Br-DMEQ; 2, PAA; 3, *m*-hydroxyphenylacetic acid; 4, PPA; 5, *p*-hydroxyphenylacetic acid; 6 and 7, the reagent blank.

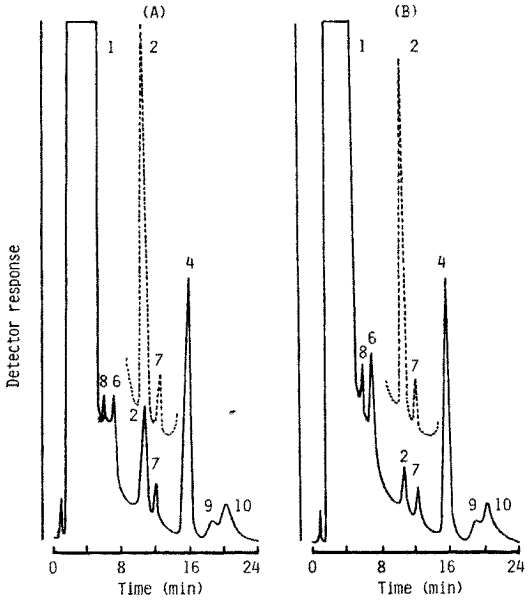

Fig. 3. Chromatograms Obtained with (A) Healthy Human and (B) Control Rat Plasma (——) before and (------) after Hydrolysis

Experimental details are described in the text. For peaks 1—7, see Fig. 2; peaks 8—10 are unidentified.

phenylpyruvic acid, which occur in biological fluids, to PAA during the procedure were observed even when they were present at unusually high concentrations in plasma (5.0 nmol/ml in plasma). Thus, further clean-up of the sample solution was not necessary.

Linearity, Detection Limit and Precision

A linear relationship was observed between the ratio of the peak height of PAA to that of PPA and the amounts of PAA (50 pmol—100 nmol) added to 50 μl of human plasma. The linear regression equation (the linear correlation coefficient in parenthesis) was $Y = 0.02053X + 0.0022$ ($r = 0.998$), where Y and X are the peak height ratio and the concentration (nmol/ml) of PAA, respectively.

The detection limit for PAA was 11 pmol/ml in plasma at a signal-to-noise ratio of 5. The sensitivity is much higher than that of the UV-HPLC method, and is comparable to those of the GC-MS methods.

The within-day precision was determined from repeated analyses ($n = 20$) of a normal human plasma containing 0.35 nmol/ml of free PAA and 2.93 nmol/ml of total PAA. The coefficients of variation were 3.9 and 3.6% for free and total PAA, respectively. The between-day precision was obtained by performing the analyses ($n = 3$ each day) using the calibration graph prepared on that day during ten days with plasma samples kept frozen at −40 °C. The coefficients of variation were 4.1 and 4.3% for free and total PAA, respectively.

PAA Concentration in Human Plasma

The levels of free, total and conjugated (calculated from differences) PAA in human plasma are given in Table I. The mean values were in agreement with those obtained by other

TABLE I. Plasma Concentrations of PAA (nmol/ml) in Normal Human

Age	Sex[a]	Free	Conjugate[b]	Total	Free/total (mol/mol)
59	M	1.81	3.09	4.90	0.37
37	M	0.49	1.77	2.26	0.22
35	M	0.44	1.45	1.89	0.23
31	M	0.34	1.49	1.83	0.18
28	M	0.45	1.42	1.87	0.24
27	M	0.54	1.63	2.17	0.25
27	M	0.67	3.03	3.70	0.22
24	M	0.57	1.71	2.28	0.25
24	M	0.52	1.55	2.07	0.25
23	M	0.37	1.72	2.09	0.18
Mean		0.62	1.89	2.58	0.24
S.D.		0.43	0.63	1.12	0.05
25	F	0.71	2.27	2.98	0.24
25	F	1.06	5.25	6.31	0.17
25	F	0.66	1.98	2.64	0.25
25	F	1.80	5.14	6.94	0.26
25	F	1.61	3.52	5.13	0.31
21	F	1.36	2.86	4.22	0.32
Mean		1.20	3.50	4.70	0.26
S.D.		0.47	1.41	1.75	0.05
Mean		0.84	2.50	3.34	0.25
S.D.		0.52	1.25	1.68	0.05

a) M, male; F, female. *b*) Conjugated PAA value is obtained by subtracting the free value from the total value.

TABLE II. Plasma Concentrations of PAA (nmol/ml) in Control Rats

	Free	Conjugate[a]	Total	Free/total (mol/mol)
1	0.23	2.13	2.36	0.10
2	0.51	3.54	4.05	0.13
3	0.32	2.83	3.15	0.10
4	0.33	2.00	2.33	0.14
5	0.47	4.06	4.53	0.10
6	0.31	1.81	2.12	0.15
7	0.48	3.12	3.60	0.13
8	0.32	2.56	2.88	0.11
9	0.31	2.46	2.77	0.12
10	0.19	1.31	1.50	0.13
Mean	0.35	2.58	2.93	0.12
S.D.	0.11	0.83	0.33	0.02

a) Conjugated PAA value is obtained by subtracting the free value from the total value.

workers.[1,2,4-6] The data indicated that plasma PAA concentration in females is significantly higher than that in males ($p<0.01$). The same observation was also reported by Davis *et al.*[2]

PAA Concentration in Rat Plasma

It is known that rats and mice, forced to swim in water, show a characteristic posture.

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