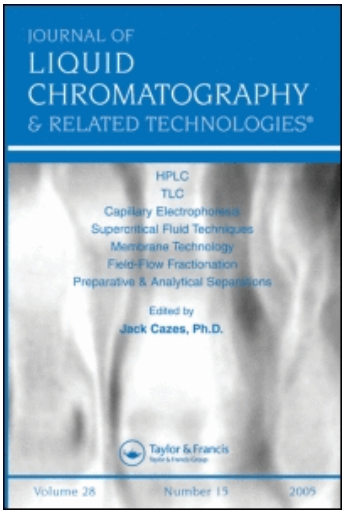

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IMPROVED METHOD FOR DETERMINATION OF β-PHENYLETHYLAMINE IN HUMAN PLASMA BY SOLID-PHASE EXTRACTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A simple, selective, and reproducible method was developed for the quantitative determination of β-phenylethylamine (PEA) in human plasma. The method involved sample clean-up procedure by a solid-phase extraction using a Sep-Pak C_{18} cartridge in the presence of phenylpropylamine (PPA) as an internal standard followed by pre-column fluorescence derivatization with *o*-phthalaldehyde, 2-mercaptoethanol. PEA and PPA were separated by reversed-phase high performance liquid chromatography (HPLC) on a C_{18} reversed-phase column with a mobile phase consisting of a 0.0375 M acetate (pH 5.5)/acetonitrile (50:50, v/v) buffer and detected fluorometrically. A linear relationship was achieved between the peak area ratios of PEA/PPA and PEA concentrations over the range of 250 to 2000 pg/injection (one injection=40 μL). The limit of detection for PEA at a signal-to-noise ratio of 3 was 4 pg/injection (100 pg/mL) in a standard solution. Total analysis was achieved in less than 25 minutes with the average PEA recov-

ery of 94.1%. The reproducibility and the repeatability of the method, assessed by calculating the mean C.V. of peak area ratio (PEA/PPA) and that of the retention times of the analytes, were in the range of 1.1-3.3% and 0.14-0.42%. The average plasma PEA level in healthy volunteers was 1129.8 ± 268.1 pg/mL (n=40, age 39.3 ± 10.3 years (mean ± S.D.)).

INTRODUCTION

β-Phenylethylamine (PEA), a trace amine, has been thought to act as a neurotransmitter or a neuromodulator in the central nervous system.[1-3] It has some properties in terms of chemical structure, pharmacological and behavioral effects in the central nerve system that are similar to those of amphetamine, a psychotomimetic.[4,5] PEA has also been related to the pathogenesis of several neuropsychiatric disorders such as schizophrenia,[6-8] depression,[9-12] other psychiatric disorders,[13,15] and Parkinson's disease.[13,14]

Several previous analytical methods, involving HPLC[11,12,,22-26] and gas chromatography-mass spectrometry (GC-MS),[6,7,11,12,14,18-21] have been developed for the assay of PEA in human plasma. However, these methods have reported different results as to the amounts of plasma PEA in patients among these disorders. These discrepancies in reported PEA levels may be in part due to the insufficiency in sensitivity, simplicity, and/or selectivity in these analytical procedures, as well as the low concentration of PEA in the biological sample.

The GC-MS methods[6,7,11,12,14,18-21] took so much analytical-cost and -time that their practical use is restricted to small numbers of sample determinations. A combination of precolumn derivatization using o-phthalaldehyde with HPLC-electrochemical or fluorescence detection was also used to determine PEA in human plasma,[7,8,17,24,25,26] human cerebrospinal fluid, and human urine.[6,10,18,23,26] However, these previous HPLC methods were not successfully available for the routine analysis of human plasma PEA owing to interference by co-eluting endogenous compounds. Therefore, much more precise clean up procedures are required, instead of the previous solvent or solid phase extraction methods.

The purpose of this study was to develop a simple and practicable method applicable in a routine auto-sampler procedure, using o-phthalaldehyde/(2ME) as a fluorescence derivatization reagent for amines and Sep-Pak C_{18} cartridge for the purpose of removing the contaminants. PPA, not a biogenic amine, was used as the internal standard (I.S.) in this procedure. The chemical structures of these analytes, and AMP, are shown in Figure 1.

The present study was approved by the local ethics committee and all subjects gave their written informed consent to participate in the study.

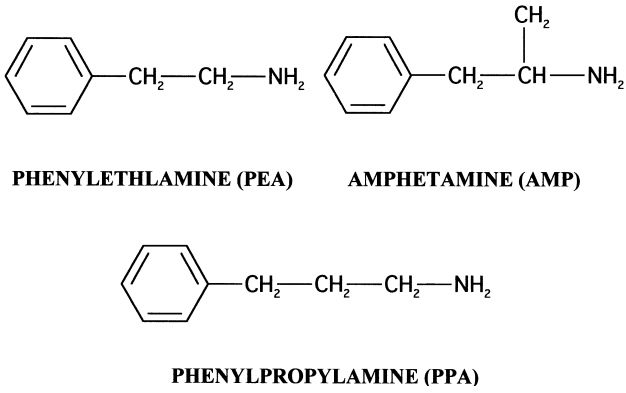

Figure 1. Chemical structures of analytes and AMP.

EXPERIMENTAL

Chemicals and Materials

OPA and 2-ME were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PEA and phenylpropylamine (PPA) were from Aldrich Chemical Co. (Milwaukee, WI, USA). All other reagents and solvents were supplied by Wako Pure Chemical Industries (Osaka, Japan). A Sep-Pak C_{18} cartridge and a Guard-Pak Inserts Resolve C_{18} guard column were obtained from Waters Assoc. (Milford, MA, USA). Distilled water, purified using a Toray Pure LV-08 system (Toray Co., Tokyo, Japan), with a resistance of greater than 18.3MΩ x cm, was used for the preparation of the buffers. PEA and PPA solutions stored in a stable environment at 4°C were used within a month. An OPA-2ME derivative solution, also stocked at 4°C, was used within a week. All working solutions were prepared daily by diluting the stocked standard solutions.

Chromatographic Conditions

The HPLC system consisted of a Gilson model 305 pump (Gilson, Middleton, WI, USA), a Gilson Model 231 auto-injector fitted with a 50 µL sample loading loop, and an Eicompak CA-5ODS C_{18} column (150 x 4.6 mm I.D.; 5 µm particle size, 120 Å pore size, Eicom Co., Kyoto, Japan) with a Guard-Pak Inserts Resolve C_{18}guard column.

Plasma PEA and PPA were detected using a Gilson model 121 fluorometer equipped with a 9 µL flow-cell and an EPZ/DJT halogen lump (13.8V, 50W, Philips, Aachen, Germany). The mobile phase consisted of a 0.0375 M acetate

(pH 5.5)/acetonitrile (50 : 50, v/v) buffer and was degassed using an ERC-3000 degasser (Erma Co., Tokyo, Japan). The separation was performed at a flow rate of 1.2 mL/min, which corresponded to a back-up pressure of about 7.8M Pa. The Chromatograms were recorded and integrated using a C-R4A Chromatopac system (Shimadzu, Kyoto, Japan).

Samples Preparation

Fresh blood samples were collected from the antecubital veins of 40 healthy volunteers (age, mean ± S.D.: male (n=20); 38.9 ± 9.9 years, female (n=20); 39.7 ± 10.9 years) in the morning (7:00 AM-10:00 AM) after an overnight fast. Samples were collected into 5 mL vials and centrifuged at 3,000 rpm for 15 min to separate plasma after standing on ice for 10-30 min.

Following separation, all plasma samples were removed, then frozen immediately and stored at -80°C until analyzed. All subjects were not treated with any drugs. 1000 pico-gram of PPA was added to 1 mL of each plasma sample as the I.S. and deproteinized by adding 1 mL of 5% trichloroacetic acid. Following vortexed-mixing for 30 seconds, after standing for 5 min, the mixture was centrifuged at 3,000 rpm for 15 min again. Then the supernatant was removed as a biological sample.

Sample Clean-Up and Extraction

The present solid-phase extraction procedure of PEA from human plasma was performed by modifying the method developed by Lauber et al.[21] The pH of the deproteinized supernatant was adjusted to over 13 by adding 300 mL of 5M potassium hydroxide. After pretreatment of a Sep-Pak C_{18} cartridge by washing with 5 mL of 30% methanol and 5 mL of deionized water (twice) successively, the sample solution was passed through the cartridge and analytes were retained in it. Then the cartridge was rinsed with 3.5 mL of 0.1N HCl to remove chemical contaminants, thereby improving the recovery of these trace amines.

PEA and PPA were eluted with 1 mL of methanol from the cartridge and the eluate was collected in a 5 mL glass tube. Then, the solution was evaporated to dryness under a blow of dry nitrogen gas in a water bath at 45°C and the sample residue was redissolved in 100 μL of methanol. The extracted solution was subsequently poured into a Millipore Ultrafree-MC 0.45 μm filter unit (Millipore Corp., Bedford, MA, USA) and centrifuged at 3,000 rpm for 5 min to remove contaminants. The 40 μL of resulting solution was used for the derivatization.

Derivatization Procedure

OPA reagent solution containing 54 mg of OPA in 10 mL of ethanol/0.1M sodium tetraborate buffer (1:9, v/v) was prepared and diluted 1:10 to a concen-

tration of 4 mM OPA with 0.1 M sodium tetraborate buffer. To 1 mL of the OPA solution was subsequently added the 4 μL of 2-ME.

After mixing 10 μL of the OPA-2ME derivative reagent solution with the 40 μL of sample solution three times, 50 μL of the mixture was allowed to react for 1.0 min. Then 50 μL volume of the reactant was injected into the column using the Gilson Model 231 auto-injector.

Quantitative Determination

Peak identification, peak area quantification, and integration were performed using a Shimadzu C-R4A Chromatopac system. PEA was identified by its retention time relative to the reference peak of PPA. The amounts of PEA were calculated based on the external standard method. Essential factors to improve the resolution between PEA and PPA were the composition and pH of the buffer, in addition to the clean up procedure for plasma samples; so the mobile phase was made up of acetonitrile/0.0375 M acetate in the solvent to 50:50 (v/v) and was adjusted to a pH of 5.5 with acetic acid. The detection limit was evaluated by analyzing five replicates of the lowest calibration standard.

Linearity

The calibration curve was constructed by plotting the peak-area ratio of PEA to the I.S. against PEA concentrations ranging from 62.5-2000 pg/injection. Each concentration consists of five repeated injections using the standard solutions. Quantitative determination for plasma PEA was done by measuring the peak area ratios of PEA to the I.S..

Reproducibility and Recovery

The reproducibility of the method was also determined by analyzing five replicates of standard solutions spiked with known amounts of PEA. The extraction recovery was assessed by comparing of spiked PEA concentrations ranging from 250-2000 pg/mL with five replicates assay at each concentration in plasma, with that of blank plasma.

RESULTS AND DISCUSSION

Chromatograms and Specificity

Figure 2 shows representative chromatograms obtained from a standard solution without treatment of extraction spiked with PEA (1000 pg/mL) and

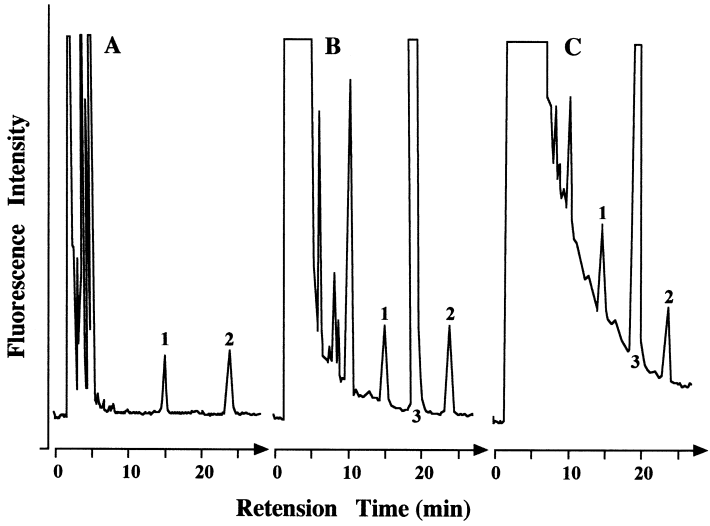

Figure 2. Representative HPLC-FD chromatograms of the OPA-2ME derivatives of PEA and PPA (I.S.; 1000 pg/mL). (A) A standard solution without treatment of extraction, containing PEA (1000 pg/mL) and PPA (I.S.). (B) A standard solution using a treatment of extraction, containing PEA (1000 pg/mL) and PPA (I.S.). (C) A blank human plasma containing PPA (I.S.). I.S.: internal standard, Peaks : 1 = PEA ; 2 = PPA ; 3 = reagent blank.

PPA (I.S; 1000pg/mL) (Figure 2A), a standard solution using a treatment of extraction spiked with PEA (1000 pg/mL) and the I.S. (Figure 2B), and 1 mL of blank human plasma spiked with the I.S. (Figure 2C). Although, a few endogenous plasma components reacted with OPA-2ME derivatives to give fluorescent compounds, they did not interfere with the determination of the analytes on the retention times.

Derivatization Conditions

OPA-2ME derivatives gave the most intense and constant peaks for PEA and PPA, more than a concentration of 2 mM OPA; thus, a concentration of 4 mM OPA was chosen for the derivatization reagent in this method. In addition, the derivatization reaction was complete within 30 seconds, independent of the operating temperature ranging from 15-30°C. Therefore, reaction standing for 1 min at room temperature was used in the procedure.

Determination of PEA in Human Plasma

The components of peaks 1 and 2 in Figure 2 were identified as OPA-2ME derivatives of PEA and PPA, on the basis of the retention times of the their peaks in comparison with those of the standard compounds. The precision of the method was evaluated in the measurement of the retention time by analyzing five replicates of a standard mixture of PEA and PPA for seven days. The retention times of analytes were 14.8 min for PEA and 24.2 min for PPA at a flow rate of 1.2 mL/min (Table 1).

The average repeatability of PEA and PPA shown in Table 1 were 0.30% and 0.27% in C.V. (ranging from 0.16%-0.42% and 0.14-0.40%). No peaks were observed in the chromatogram at the retention time for the analytes when the derivatization reaction was not performed. They showed the fluorescence detection performed at excitation and emission wavelength of 440 nm and 340 nm, respectively, with sensitivity setting at 0.002 AUFS.

Linearity and Detection Limit

A linear relationship was observed between the peak area ratios of PEA and that of the I.S. as shown in Figure 3. The standard calibration curve of PEA

Table 1

Retention Time, Standard Deviation, and Coefficient of Variation of PEA and PPA Performed in Seven Days*

	R.T. of PEA[a]			R.T. of PPA[b]		
Day	Mean (min)[c]	S.D.	C.V. (%)	Mean (min)	S.D.	C.V. (%)
1	14.844	0.027	0.18	24.159	0.035	0.14
2	14.828	0.063	0.42	24.199	0.069	0.29
3	14.831	0.024	0.16	24.147	0.049	0.20
4	14.878	0.049	0.33	24.221	0.083	0.34
5	14.837	0.061	0.41	24.149	0.045	0.19
6	14.858	0.047	0.32	24.214	0.044	0.18
7	14.850	0.039	0.26	24.163	0.097	0.40

* Rentention time = R.D., Standard deviation = S.D., Coefficient of Variation = C.V.. [a]The average R.T. of PEA = 14.847 min. The average C.V. of PEA = 0.30. [b] The average R.T. of PPA = 24.178 min. The average C.V. of PPA = 0.27.

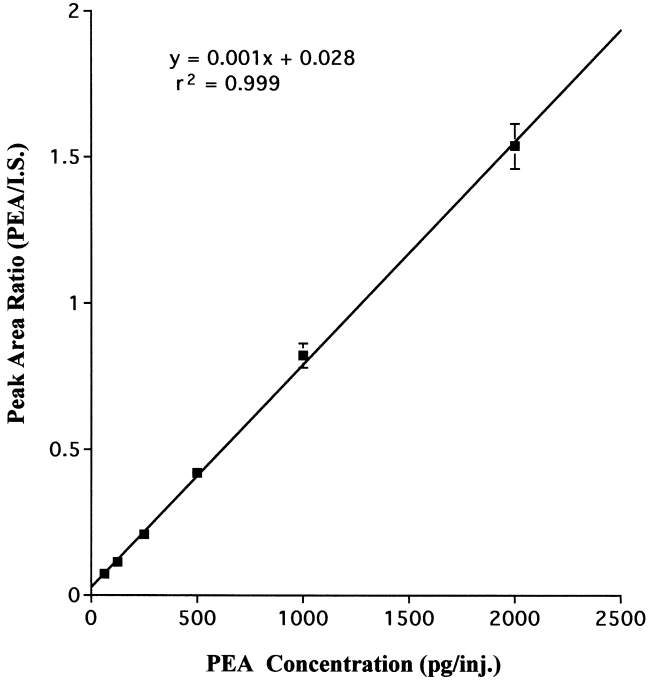

Figure 3. Relationship between peak area ratio (PEA/PPA) and PEA concentration. The samples were separated using the C_{18} reversed-phase column (150 x 4.6 mm I.D.; 5 μm particle size) and determined by using the Gilson model 121 fluorometer equipped with a 9 μL flow cell and an EPZ/DJT halogen lump as described in the text.

showed good linearity, with the correlation coefficient of 0.999 in the practical working concentration ranging from 62.5-2000 pg/injection containing 1000 pg/mL of PPA as the I.S.. Calibration curve for PEA was given as follows: y = 0.0001x + 0.028, where y is the peak area ratio of PEA to I.S. and x is the concentration of PEA in pg/injection.

The lowest detection limit for PEA was 100 pg/mL (4 pg/injection) in standard solution at a signal-to-noise ratio of 3. This sensitivity was relatively high compared with those of the reported fluorometric methods.[17,24,25]

Reproducibility and Recovery

The reproducibility of the procedure using unextracted standard solutions is shown in Table 2. The intra- and inter-day reproducibility of the method was

Table 2

Reproducibility of Peak Area Ratio for Unextracted Added PEA Concentrations in Standard Solutions

Added PEA Conc. (pg/inj.)[a]	Intra-Day Reproducibility Peak Area Ratio[b] Mean (n=5)	S.D.	C.V. (%)	Inter-Day Reproducibility Peak Area Ratio[b] Mean (n=5)	S.D.	C.V. (%)
250	0.226	0.007	3.3	0.223	0.006	2.8
500	0.416	0.010	2.4	0.422	0.013	3.1
1000	0.837	0.009	1.1	0.838	0.012	1.5
2000	1.642	0.018	1.1	1.646	0.020	1.2

[a] One injection = 40 μL. [b] Peak area ratio of added PEA to PPA (I.S.; 1000 pg/inj.).

established by analyzing five replicates of standard solutions ranging from 250-2000 pg/injection of PEA. a) The mean standard deviations (S.D.) of reproducibility were 0.7%, 1.0%, 0.9%, and 1.8% in intra-day, and 0.6%, 1.3%, 1.2%, and 2.0% in inter-day. b) The reproducibility showed very little variation with a C.V. of 3.3%, 2.4%, 1.1%, and 1.1% in intra-day, and 2.8%, 3.1%, 1.5%, and 1.2% in inter-day.

The extraction recoveries of PEA (added 250 pg, 500 pg, 1000 pg, and 2000 pg of PEA to 1 mL of plasma samples) were 94.7 ± 9.7%, 93.5 ± 11.9%, 93.2 ± 7.8%, and 94.8 ± 8.3 (mean ± S.D., n=5), respectively (Table 3). The average extraction recovery of PEA was 94.1%, which was better than those found in the previous literature.[17,24,25]

PEA Concentration in Human Plasma and Comparison with Other Techniques

The data in Table 4 provides a comparison of the mean plasma PEA level of the present assay with those determined by several researchers; and the reported PEA concentrations differ considerably. The mean plasma PEA concentration from 40 fasting healthy volunteers was 1129.8 ± 268.1 pg/mL (n=40, age 39.3 ± 10.3 years (mean ± S.D.)). The value by our method was relatively high compared with those of other laboratories (the average plasma PEA concentration by the previous GC-MS and HPLC methods was 651.8 pg/mL,

Table 3

Recovery of Plasma PEA

Added PEA Conc.[a] (pg/mL)	Determination Mean (pg/mL)[c]	S.D.	Recovery[b] Mean (%)[c]	S.D.	C.V. (%)
0	1125	8.6	----	----	----
250	1356	36.5	94.7	9.7	10.2
500	1594	59.4	93.5	11.9	12.7
1000	2056	75.1	93.2	7.8	8.4
2000	3018	171.5	94.8	8.3	8.8

[a] Addition of each PEA concentration to 1 mL of plasma sample. [b] The average plasma PEA recovery = 94.1%. [c] n = 5.

Table 4

Plasma PEA Concentration in Healthy Volunteers Obtained by the Present HPLC Method and Other Methods

Reference	Analytical Method	PEA Conc.[a] (pg/mL) Mean	S.D.	Number of Subject[b]
(7)	GC-MS	90.2	40.0	17
(12)	GC-MS	1190.0	310	32
(19)	GC-MS	835.5	63.1	15
(17)	HPLC (precolumn)	335.0	255	12
(24)	HPLC (precolumn)	52.0	36.3	16
(25)	HPLC (precolumn)	930.1	362.6	10
	Present method	1129.8	268.1	40

[a] The average plasma PEA conc. of these analytical methods = 651.8 pg/mL [b] (n = 20.3).

n=20.1),[7,12,14,19,24,25] especially, higher than those obtained by the HPLC methods (the average plasma PEA concentration was 439.0 pg/mL, n=12.7).[12,24,25]

CONCLUSION

The described HPLC method demonstrated the advantage of the clear resolution and the high recovery, in addition to the repeatability and reproducibility for the plasma PEA analysis: it could improve the recovery of plasma PEA due to remove various endogenous contaminants issued from the analytical procedure. Also, it enabled us to separate and quantify plasma PEA and PPA in less than 25 min within 30 min run time, and to perform as many as 40 successive runs. Therefore, the presented procedure should be applicable to the routine analysis of a large number of plasma PEA in medical and biological investigations.

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