Current Topics

Applications of Fluorescence Detection in Current Pharmaceutical Research

Regular Article

Liquid Chromatographic Determination of *o*-Phosphoethanolamine in Human Plasma Using Fluorescent Derivatization

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Received June 23, 2022; accepted August 20, 2022

In this study, an HPLC analysis method using pre-column derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was developed for the determination of *o*-phosphoethanolamine (PEA), which is a potential biomarker for the diagnosis of major depressive disorder, in human plasma sample. After PEA was derivatized with AQC under mild conditions, the obtained derivative was subjected to purification with a titanium dioxide-modified monolithic silica spin column (MonoSpin® TiO). The eluate from the MonoSpin® TiO was directly injected into an amide-type hydrophilic interaction liquid chromatography (HILIC) column-equipped HPLC system, and the resulting derivative could be separated on the column under alkaline mobile phase conditions and subsequently detected fluorometrically at excitation and emission wavelengths of 250 and 395 nm, respectively. The limit of detection and limit of quantification for a 10 μL injection volume of PEA were 0.052 and 0.17 μM, respectively. The method was validated at 0.2, 1.0, and 5.0 nmol/mL levels in plasma sample, and the precision values were 2.0–6.6% as relative standard deviation and the correlation coefficient (*r*) of the calibration curve was 0.9995. Furthermore, applicability of this method was demonstrated by analyzing PEA levels in plasma samples from mental illness patients.

Key words *o*-phosphoethanolamine, fluorescence derivatization, hydrophilic interaction liquid chromatography (HILIC), human plasma

Introduction

Major depressive disorder (MDD) is a common mental illness with a worldwide prevalence of 6 to 8%.[1,2] The diagnosis of MDD primarily depends on the subjective evaluation of depressive symptoms, and it may also cause misdiagnosis as bipolar disorder (BD) because of their similarity in clinical symptoms.[3,4] Therefore, establishing the blood-based biomarkers for depressive disorder have attracted attention in psychiatric research to facilitate the diagnosis, selection of a treatment, and prediction of the treatment response. Recently, Kawamura *et al.* showed that the plasma levels of *o*-phosphoethanolamine (PEA) in MDD patients was different from that in healthy controls.[5,6] Hence, it is thought that determining the PEA concentrations in plasma might be a useful biomarker for MDD diagnosis. Several methods for measuring PEA with enzymatic measurements,[7] capillary electrophoresis (CE) with UV[8] or laser-induced fluorescence detection,[9] GC-MS,[10] and LC-MS[11–14] have been reported. Although the chromatography with MS detection methods is highly sensitive and selective, their apparatus and operating costs are too expensive for routine analyses. On the other hand, post-column fluorescence derivatization method for PEA with *o*-phthalaldehyde was also reported[15,16] However, the post-column methods have some drawbacks, which are complicated equipment and high consumption of reagents and solvents, and therefore more affordable and convenient method is required in the analysis of endogenous PEA in biological samples.

In this study, we developed simple and cost-effective analysis method for PEA using pre-column fluorescence derivatization approach and analysis by LC with fluorescence detection. We employed 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) as commercially available fluorescence pre-column derivatization reagent (AccQ·Tag™ Ultra reagent), which enables easy and rapid derivatization of amino group, in this study (Fig. 1). Furthermore, for purification of PEA derivative from by-products and/or other AQC derivatives from biological matrix, the solid phase extraction with a titanium dioxide (TiO_2)-modified monolithic silica spin column, which has a binding affinity for phosphorus compounds,[17,18] was applied after derivatization. Subsequently, the obtained derivative of PEA was analyzed with hydrophilic interaction liquid chromatography (HILIC). The HILIC has been employed in the effective analysis of various highly polar compounds.[19,20] Among various HILIC column, the amide-type column can be used at high pH values to obtain moderate retentions and separations of negatively charged phosphates under alkaline mobile phase conditions on the column.[21–23] Therefore, in this study, we optimized the separation conditions of the PEA derivative with the amide-type HILIC column.

After optimization study of derivatization, extraction and

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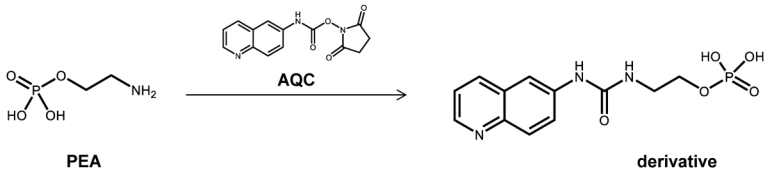

Fig. 1. Fluorescence Derivatization of PEA with AQC

HPLC analysis conditions, the PEA concentrations in plasma samples obtained from healthy controls and patients with MDD and BD were measured to confirm the feasibility of the method.

Experimental

Reagents and Chemicals PEA was purchased from Tokyo Chemical Industry (Tokyo, Japan). The AccQ·Tag™ Ultra derivatization kit (AccQ·Tag™ Ultra reagent powder, borate buffer, and reagent diluent) was purchased from Waters Corporation (Milford, MA, U.S.A.). Pooled human plasma was obtained from Cosmo Bio (Tokyo, Japan). Ultrapure water purified using a Milli-Q gradient system (Millipore, Billerica, MA, U.S.A.) was used for preparing all aqueous solutions. All other reagents and solvents from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) were of LC-grade or the highest purity and used as received.

A working standard solution were prepared by diluting a standard stock solution (10 mM) of PEA prepared in ultrapure water to the required concentrations with ultrapure water before use.

A MonoSpin® TiO column was obtained from GL Sciences Inc. (Osaka, Japan) and pre-conditioned with 300 μL of 90% acetonitrile containing 300 mg/mL-lactic acid and 0.05% trifluoroacetic acid before use.

Plasma Samples from Patients Plasma samples were obtained from 10 MDD patients, 4 BD patients, and 10 healthy subjects in Saga University Hospital. An ethylenediaminetetraacetic acid (EDTA)-containing blood collection tube (Terumo Venoject II evacuated tube, Tokyo, Japan) was used for the collection. The obtained plasma was transferred to 1.5 mL screw-cap tubes and stored at −80 °C before use. To 100 μL of the plasma sample, 100 μL of borate buffer was added. After vortex mixing for a few seconds, the mixture was ultra-filtered by Amicon ultracel-3K Membrane (Millipore Corporation, Bedford, MA, U.S.A.) with centrifugation at 18000 × ***g*** for 10 min. The filtrate (40 μL) was subjected to derivatization using the procedure outlined below.

The present experiments were approved by the Ethics Committee of the Central Research Institute of Fukuoka University (approval number: U20-10–001) and Saga University Hospital (approval number: 2020-05-02), and performed in accordance with established guidelines.

Derivatization Procedure Derivatization of PEA was performed with the AccQ·Tag™ Ultra derivatization kit. To 40 μL of sample in a 1.5-mL polypropylene tube, 15 μL of borate buffer and 25 μL of 10 mM AQC reagent solution were added. The tube was then allowed to stand at room temperature for 5 min. After 470 μL aliquot of acetonitrile containing 300 mg/mL-lactic acid and 0.05% trifluoroacetic acid was added to the reaction mixture, the resulting solution was subjected to purification with MonoSpin® TiO column.

Purification with Monospin TiO Sample solution obtained after derivatization was loaded onto the preconditioned MonoSpin® TiO column. The column was then washed with 300 μL of 50% acetonitrile containing 0.05% trifluoroacetic acid. After this, the derivative on the column were eluted with 80 μL of 80% acetonitrile containing 4% ammonia. The eluate was directly injected into the HPLC system. All procedure of this column were performed by centrifugation at 2000 × ***g*** for 1 min.

Instrumentation and Their Conditions A Waters Alliance™ 2695 system (Waters, Milford, MA, U.S.A.) consisting of a binary pump, an on-line degasser, an autosampler, and a column oven and a Waters 2475 fluorescence detector equipped with an 8 μL flow cell were used. Injections of 10-μL samples were carried out automatically. A XBridge Amide column (150 × 2.1 mm i.d., particle size 3.5 μm, Waters) was used. The fluorescence detector was operated at an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Mobile phase A (10% ammonia solution and acetonitrile (10 : 90, v/v)) and mobile phase B (60 mM ammonium carbonate buffer (8% ammonia, pH 10.0) and acetonitrile (25 : 75, v/v)) were used as the mobile phases for the gradient elution (gradient curve: 0–1.2 min, 10% B; 1.2–5 min, linear gradient from 10% B to 100% B; 5–15 min, 100% B; 15–20 min, linear gradient from 100% B to 10% B; run-time, 30 min). The flow rate and column oven temperature were set at 0.2 mL/min and 45 °C, respectively.

Validation Study The limit of detection (LOD) and limit of quantification (LOQ) of PEA in the present method were defined as the concentrations that gave signal-to-noise ratios (*S*/*N*) of 3 and 10, respectively. Calibration curves for determination of the PEA quantities in human plasma were obtained from the spiked-pooled human plasma samples measured at concentrations of 0.2, 0.5, 1.0, 2.5, and 5.0 nmol/mL plasma. The calibration samples were subjected to the present derivatization with AQC and purification with MonoSpin® TiO procedures after preparation. To evaluate the accuracy of the method, spiked pooled human plasma samples (0.2, 1.0, and 5.0 nmol/mL plasma) were analyzed. The accuracy was determined by comparing the responses obtained from spiked pooled human plasma samples and those from standard solutions of the same concentrations. The intra-day and inter-day precisions of the method were evaluated analyzing three replicates of standard solutions at three concentration levels (0.2, 1.0, and 5.0 nmol/mL plasma) on three different days. Furthermore, to evaluate the freeze-thaw cycle stability, samples were stored for at least 24 h at −80 °C followed by thawing at room temperature for three cycles, and the samples were compared with a reference sample that was prepared just before the analysis.

Results and Discussion

Derivatization and Purification Conditions Derivatization was successfully performed with AccQ·Tag kit. We

examined the effects of reaction time (0–60 min) and temperature (25–80 °C) to find the optimal derivatization conditions. A reaction time of more than 5 min was required to obtain the largest peak area, and the reaction was not affected by temperature. Therefore, reaction time of 5 min at room temperature was the optimal condition for the derivatization of PEA. The derivative was stable for at least 1 d in the autosampler at room temperature. Furthermore, after derivatization, the extraction with monolithic spin column (MonoSpin® TiO) was applied for purification of the PEA derivative from sample matrix, such as excess reagent and/or non-phosphorus derivatives, to exclude interference peaks on the chromatogram. The affinity of titanium dioxide-modified column, such as the ability of ligand for Lewis bases, has been applied as selective enrichment and/or purification technique for phosphate-containing compounds.[17,18] Using this column, the retention of phosphate compounds including PEA can be easily achieved under acidic condition in the presence of 2,5-dihydroxybenzoic acid or aliphatic hydroxy acids, whereas other acidic compounds such as carboxylic acids are not retained under the same conditions. In this study, the derivative of PEA was also successfully applied to the pre-conditioned MonoSpin® TiO under the acidic conditions with lactic acid and trifluoroacetic acid. The elution could be performed with ammonia hydroxide in aqueous acetonitrile solution. Although the recovery of PEA derivative after this extraction was relatively low (approximately 50%), the quantification of PEA using this method is acceptable because its reproducibility was good and the calibration standards for determination of the PEA in human plasma were obtained from the same derivatization and purification procedure.

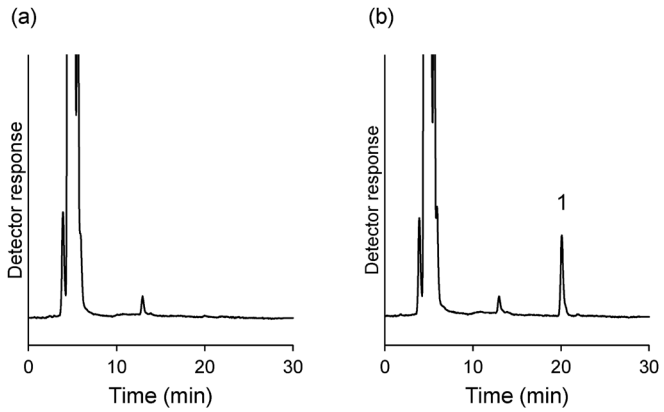

Fig. 2. Typical Chromatograms of (a) Blank and (b) 1.0 μM Standard Solution

Analysis of Standards Figure 2 shows typical chromatograms obtained from analysis of the standard of PEA with the present method. We performed the LC separation for the PEA derivative using an amide-type HILIC column, which is stable to a wide pH range of mobile phase. The HILIC analytical conditions for the PEA derivative in this study were adopted from the method published previously.[24,25] Under alkaline mobile phase conditions, not only the phosphate-containing compounds are easily deprotonated, but also the resulting negatively charged can be strongly retained on the HILIC column. In this study, ammonium bicarbonate was also added as an alkaline salt in mobile phase, and we found that a concentration of 60 mM ammonium bicarbonate was optimal for analysis of the PEA derivative under HILIC separation conditions.

Under the optimized conditions, the LOD and LOQ for PEA were 0.052 and 0.17 μM (corresponding to 0.13 and 0.44 pmol on column), respectively. The obtained LOQ was almost identical or lower than those from previously reported methods (0.038–10 μM).[11–14]

Analysis of Pooled Human Plasma Samples To investigate the applicability to biological sample, the present method was applied to the analysis of pooled-human plasma samples spiked with PEA standard. Figure 3 illustrates typical chromatograms obtained from spiked and non-spiked pooled-human plasma samples. As expected, the PEA derivative was successfully separated from the matrix components of plasma. Therefore, this method enables the selective analysis of PEA in biological samples. The validation data for the spiked-plasma samples are presented in Table 1. The correlation coefficient of the calibration curve obtained from standard spiked plasma in the concentration range of 0.2–5 nmol/mL plasma was 0.9995. The accuracy in the range of 95–107% was achieved, and the intra-day and inter-day precisions were within 6.3 and 6.6%, respectively. Moreover, the stability of PEA derivatives after freeze-thaw cycles was 92–105% at 0.2–5 nmol/mL plasma range, indicating the freeze-thaw process had little effect on the quantification of PEA.

Application to Clinical Samples We applied this method to the analysis of plasma samples obtained from MDD, BD, and healthy control subjects. Figures 4 and 5 show typical

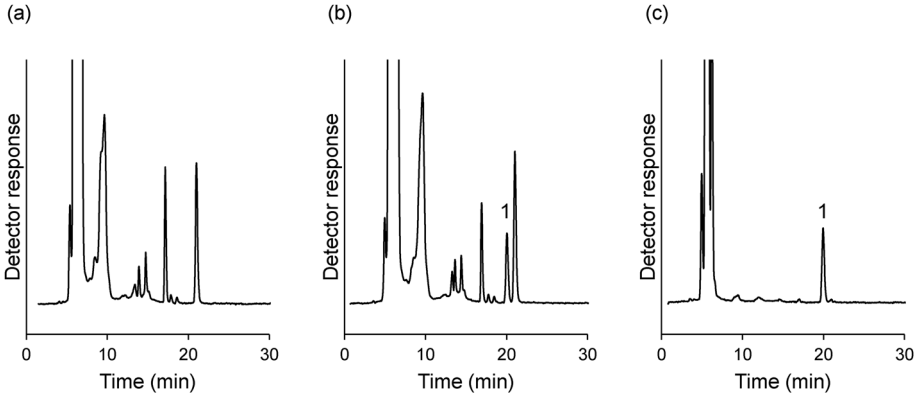

Fig. 3. Typical Chromatograms Obtained from the Analysis of (a) Non-spiked Plasma (b) 1.0 nmol/mL Standard Spiked Plasma, and (c) 1.0 μM Standard Solution

Table 1. Validation Data Obtained from Spiked-Human Plasma Samples

Spiked concentration (nmol/mL plasma)	Accuracy[a] (%)	Intra-day precision[b] (%, $n = 3$)	Inter-day precision[b] (%, $n = 3$)	Stability[c] (%)
0.2	102	5.2	6.6	100
1.0	107	6.3	6.6	92
5.0	95	5.0	6.2	105

a) Percent ratio of response of spiked pooled human plasma and that of standard of the same concentration. *b*) Relative standard deviations of peak areas. *c*) Percent ratio of peak-area after three freeze–thaw cycles.

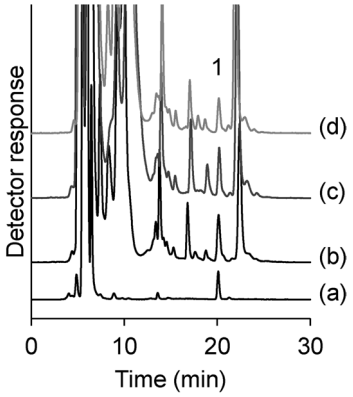

Fig. 4. Typical Chromatograms Obtained from PEA in a (a) Standard Solution, (b) Healthy Control, (c) MDD Patient, and (d) BD Patient

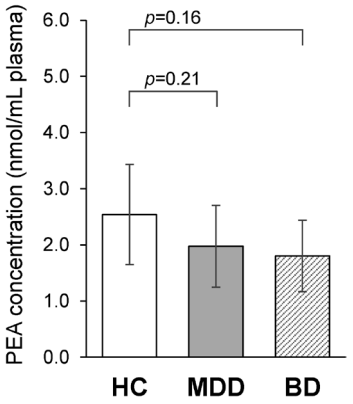

Fig. 5. PEA Concentration in Plasma Obtained from the Healthy Controls (HC, $n = 10$), MDD Patients ($n = 6$), and BD Patients ($n = 4$)

Results are expressed as the mean ± standard deviation.

chromatograms and measured concentrations obtained from each plasma sample. In this study, the measured concentration in healthy controls of PEA was 1.4–4.3 nmol/mL plasma (mean concentration: 2.5 nmol/mL plasma). In contrast, for patients with MDD and BD, the PEA concentrations were 1.3–3.3 nmol/mL plasma (mean concentration: 2.0 nmol/mL plasma) and 1.3–2.7 nmol/mL plasma (mean concentration: 1.8 nmol/mL plasma), respectively. Consequently, the levels of PEA in plasma were slightly lower in both patients compared with healthy controls. Although no significance was observed among the groups (Fig. 5), the results obtained from MDD were identical to those from previous reports,[5,6] although the PEA concentrations from BD were same levels to those from MDD. This may be due to few patients number. Further study would be needed continuously to gain more insight into these results.

Conclusions

A fluorogenic derivatization method for the HPLC analysis of PEA was developed. After PEA was derivatized with AQC, the obtained derivative was purified using a TiO_2-modified monolithic silica spin column. Furthermore, the derivative was successfully retained on an amide-type HILIC column and subsequently detected fluorometrically at enough sensitivity. This method enabled the analysis of PEA in human plasma, and it can also perform the determination of concentrations of PEA in human plasma obtained from MDD and BD patients. Therefore, the proposed method is a beneficial and useful analytical tool for the diagnosis of depressive disorders by analyzing PEA in human plasma.

Acknowledgments We extend thanks to Ms. M. Nakamura and Ms. T. Sakamoto (Faculty of Pharmaceutical Sciences, Fukuoka University) for their excellent technical assistance. This work was supported in part by a Grant-in-Aid for Scientific Research (C) [Grant number 21K12718] from the Japan Society for the Promotion of Science.

Conflict of Interest The authors declare no conflict of interest.

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