Development of Highly Sensitive Analytical Method for Evaluation of Evening Primrose Oil's Enhancing Effect in Prostaglandin E₁(OP 1206) Biosynthesis

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ABSTRACT

This study aimed to develop and validate highly sensitive determination method of a prostaglandin (PGE₁, OP 1206) in human plasma by LC-MS/MS using column switching. Plasma stored at -30°C and treated with methanol effectively inhibited interferences synthesized post-sampling. Samples were added with internal standard and were separated by reversed-phase HPLC with a cycle time of 30min. The method was selective for OP 1206 and the regression models, based on internal standard, were linear across the concentration range 0.5-50 pg/mL with the limit of quantification of 0.5 pg/mL (limit of quantitation, LOQ) for OP 1206. The calibration curve of OP 1206 standards spiked in five individual plasma samples was linear ($r^2=0.9999$). Accuracy and precision at the concentrations of 0.5, 1.5, 5.0 and 40 pg/mL, and at the lower LOQ of 0.5 pg/mL were excellent at < 20%. OP1206 was stable in plasma samples for at least 24 hours at room temperature, 24 hours frozen at -70°C, 24 hours in an auto sampler at 6°C, and for two freeze/unfreezing cycles. The validated determination method successfully quantified the concentrations of OP 1206 in plasma samples from simulated administering a single 5 µg OP 1206 formulation. Thus, this novel LC-MS/MS technique for drug separation, detection and quantitation is expected to become the standard highly-sensitive detection method in bioanalysis and to be applied to many low dose pharmaceutical products.

Keywords: analytical validation, evening primrose oil, LC-MS/MS, Prostaglandin E₁

Introduction

Evening primrose is native to Central America, and is the most widely used wild medicinal plant around the world. Oil extracted from evening primrose has long been used to treat eczema, asthma, rheumatthritis, premenstrual syndrome (PMS), and menopausal symptoms, and every substance found in the extracted oil is known to be anti-inflammatory (Wettasinghe et al., 2002). The oil extracted from the seeds of evening primrose has a high content of Y-linolenic acid (about 10%, w/w) and about 1% linolenic acid, a precursor of prostaglandin, which has been reported to be effective in treating a variety of diseases (Rodgers et al., 2009). In addition, prostaglandins extracted from various medicinal plants (Zimmerman and Feng, 1978) are bioactive, and thus have been used as an active ingredient in medicines through

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additional studies on chemical synthesis (Tsuboi et al., 1980). General prostaglandins are composed of the 20 carbon monocarboxylic acid structure of prostanic acid with the pentagonal shape, and they are Group 1 compounds characterized by hydroxyl and keto radicals and double bonds. In terms of biological activity, prostaglandins A₂, B₂ and C₂ are effective in reducing blood pressure (Fujitani et al., 1986). Prostaglandin D₂ induces hypnosis, and prostaglandin E₂ has effects such as uterine contraction, diuresis, suppression of gastric secretion, acceleration of intestinal movement, blood pressure reduction, dilation of blood vessels and bronchial tubes, bone resorption and immunosuppression (Swainston Harrison and Plosker, 2007). Among them, prostaglandin E₁ (OP 1206) [(E)-7-[(1R,2R,3R)-3-hydroxy-2-[(3S,5S)-(E)-3-hydroxy-5-methyl-1-nonyl]-5-oxocyclopentyl]2-heptenoic acid (Figure 1) is known to be very effective in dilating blood vessels and reducing blood clots (Inoue et al., 2014). Many studies have verified the effects of prostaglandins on improvement in biosynthesis by administrating medicinal plant extracts, but it is not easy to analyze biopsy specimens in the pharmacokinetic assessment of the ingredients using general analysis methods since the content of the target ingredients is very small (Omran, 2012).

LC-MS/MS is the most widely used method of analyzing low-concentration biopsy specimens. Using this method, a liquid chromatographic ionization detector and an atmospheric pressure ionization detector are placed in the front part of the system, and two quadrupole mass filters are placed within a mass spectrometer. This system contains an interface, which increases the speed of the detection process, and provides high selectivity, specificity and analytical sensitivity (Komada et al., 2007). In addition, HPLC that uses reversed-phase columns is known as an effective method of analyzing marker substances in natural plant extracts (Lee et al., 2014).

LC-MS/MS is used as a standard method to detect and quantify substances in pharmaceutical analysis, and its application has been expanded to biopsy specimen analysis. In analyzing biopsy specimens including sample pretreatment, the high resolution of LC-MS/MS has been verified in many studies, and related studies have been actively conducted including the development of a speedy quantitative analysis method using API-LC-MS/MS (Malone and Hughes, 2014), and the investigation of metabolome (Steuer et al., 2014). In particular, technology of switching columns in LC/UV and ESI-LC-MS for microanalysis, and tandem-in-time MS have been also studied (Atkinson et al., 2015). In this regard, this study aimed to develop a method of analyzing prostaglandins within plasma through microanalysis technology, and thus to contribute to the assessment of the effectiveness of medicinal plants.

Research Methods

Reagents and solvents

OP 1206, the target substance in this study, was purchased from Yonsung Fine Chemicals (Suwon, Korea), and solvents

Figure 1. Chemical structure of OP 1206 (C₂₂H₃₆O₅).

for analysis including acetonitrile, methanol, n-hexane and ethyl acetate were purchased from Burdick & Jackson (NJ, USA). Ammonium acetate, an analysis reagent, was purchased from Sigma Aldrich (MO, USA), and 13,14- dihydroprostaglandin E1-d4, an internal standard (IS), was purchased from Cayman Chemical Company (MI, USA).

Analysis Equipment

For LC-MS/MS analysis, the Agilent 1200 Series Column Switching HPLC (Agilent, CA, USA) was used in this study for liquid chromatography and the Nanospace SI-2 3301T (Shiseido, Tokyo, Japan) pump was added. The tandem mass spectrometer used in this study was the API 4000 Q Trap (Applied biosystems, CA, USA), and the auto-sampler used to analyze samples in this study was the Agilent 1200 Series G1367B (Agilent, CA, USA). The Agilent 1200 Series G1367B (Agilent, CA, USA), a column switching valve, was also used. The columns used in the column switching system were as follows: Capcellpak MF-Ph1 (Shiseido, Tokyo, Japan) for separation; Venusil XBP C₁₈ (Phenomenex, CA, USA) for trap; and Capcellpak C₁₈ (Shiseido, Tokyo, Japan) for analysis. Data were processed using Analyst 1.4.2 Version (Applied biosystems, CA, USA).

Analysis Methods

OP 1206 was dissolved in methanol to make a stock solution (1.0 mg/mL), which was diluted with unfreezed blank plasma to make a plasma sample in which the concentration of OP 1206 was 0.5-50 pg/mL. To each stock solution of 1.5mL, 30 µg of 13,14-dihydroprostaglandin E1-d4 (30 ng/mL) was added and shaken to mix the contents for 6 seconds. These samples were loaded on the cartridge of separation columns that were stabilized with methanol and distilled water, and were cleaned with 1.5 mL of 0.2% acetic acid, n-hexane and 10% methanol respectively. After that, they were eluted with ethyl acetate 4 mL. The eluted solutions were moved to test tubes, and were vaporized in the presence of nitrogen at 55°C. Their residues were added with 180 µL of the re-dissolution solution (acetonitrile : 5mM ammonium acetate = 2 : 8, v/v), and 80 µL of the recomposed solution was collected and injected to LC-MS/MS using column switching. Based on the obtained chromatograms, the ratio of the peak area of OP 1206 to the peak area of internal standard (IS) was calculated and a calibration curve was generated. This experiment was repeated five times per day to calculate intra-day reproducibility, and was repeated for five days in a row to calculate inter-day reproducibility. A sample for producing the calibration curve was tested first, and samples of each batch were measured during the process of pretreating samples to check whether they were within the range of ±15% of theoretical values.

Mobile phase 1 for liquid chromatography analysis was obtained by mixing 1mM ammonium acetate (pH 5.5) and acetonitrile to the ratio of 57:43 (v/v), and mobile phase 2 was obtained by mixing 5 mM ammonium acetate and acetonitrile to the ratio of 70:30 (v/v). The column switching system used in this study used Capcellpak MF-Ph1 (4.6 × 50 mm, 3 µm) as separation columns, Venusil XBP C₁₈ (2.1 × 50 mm, 3 µm) as trap columns, and Capcellpak C₁₈ (1.0 × 250 mm, 3 µm) as analysis columns, and detailed analysis conditions are as shown in Table 1.

Results and Discussion

Mass Spectroscopic Analysis

The precursor ion of the target substance (OP 1206) and internal standard (13,14-dihydroprostaglandin E1-d4) was detected as [M-H]⁻ in the negative mode. The precursor ion of OP 1206 was m/z 379.2, and its production ion was m/z 299.4 ion, indicating that they were produced most at -24 eV (Figure 2). The precursor ion of 13,14-dihydroprostaglandin

Table 1. Analytical conditions of LC-MS/MS with column switching system

Parameters	Analytical conditions		
Flow rate	1) 0.055 ml/min 2) 0.6 ml/min		
Injection volume	80 μ l		
Auto-sampler temp.	10°C		
MS/MS Parameter	Negative ion mode, MRM mode		
	Compound	m/z	Collision energy (eV)
	OP 1206	379→299.4	-24.0
	13,14-dihydroprostaglandin E1-d4	359.4→241.1	-38.0
	Ion spray voltage : -4500.0 V		
	Nebulizing gas : Nitrogen gas		
	Collision gas : Nitrogen gas

Figure 2. Mass spectrum of OP 1206 precursor ion (A) and production ion (B).

E1-d4 was m/z 359.4, and its production ion was m/z 241.1 ion, indicating that they were produced most at -38 eV (Figure 3). Based on the data, each was determined as a production ion to measure after collision-induced dissociation (CID).

Results of Specificity Assessment

Samples were pre-treated using the same analysis method, and were analyzed using LC-MS/MS. The amount of time the peak of OP 1206 lasted was about 11.5 minutes, and that the peak of internal standard lasted was about 11.3 minutes. Under the analysis conditions, no other interfering substance, except OP 1206 and internal standard, was found (Figure 4).

Figure 3. Mass spectrum of internal standard precursor ion (A) and production ion (B).

Figure 4. Plasma sample chromatograms of internal standard (A) and internal standard + OP 1206 0.5 pg/mL (B).

Results of Linearity Assessment

The stock solution of 0.5 (quantitation limit), 1.5, 2.0, 5.0, 10.0 and 50.0 pg/mL was treated using the same analysis method, and was analyzed using LC-MS/MS. The equation of the calibration curve of OP 1206 obtained from plasma samples was $y = 0.0203x + 0.0149$ ($R^2 = 0.9999$), which shows very clear linearity (Figure 4). The calibration curve was

obtained using the linear regression method, and its weighted value was obtained using $1/x^2$ (Figure 5).

Results of Accuracy, Precision and Quantitation Limit Assessment

The OP 1206 plasma stock solution of 0.5, 1.5, 5 and 40 pg/mL were used to assess accuracy and precision, and accuracy ($n=5$) was calculated as a percentage (%) by dividing the average value of the concentration, quantified according to the calibration curve, by known concentration. Precision ($n=5$) was calculated as a percentage (%) by dividing the standard deviation of the ratio of the peak area of OP 1206 to the peak area of internal standard by the average value of the ratio of the peak area of OP 1206 to the peak area of internal standard. This experiment was repeated five times per day to calculate intra-day reproducibility (CV%), and was repeated for five days to calculate inter-day reproducibility (CV%). The limit of quantitation (0.5pg/mL, LOQ) was a concentration of which signal to noise ratio was 5% or higher, precision was 20% or lower, and accuracy was between 80% and 120%.

The intra-day and inter-day precision (CV%) of the analysis method used in this study was 15% or lower, and the intra-day and inter-day precision at the lower quantitation limit was 20% or lower. The intra-day accuracy was 85.77 ~ 97.57%, and the intra-day accuracy at the quantitation limit was 90.60%. The inter-day accuracy was 95.02 ~ 111.60%, and the inter-day accuracy at the quantitation limit was 106.8%. The sensitivity of the analysis method was high (0.5 pg/mL) (Table 2).

Results of Solution Stability Assessment

All the stability assessment on OP 1206, the target substance in this study, was conducted at a low (1.5 pg/mL) and high (40 pg/mL) concentration. Since the stability of the stock solution for OP 1206 and inter standard was already secured, it was not tested. The stability of test solutions that had not been established was assessed by dividing it into short-term temperature stability, freezing/unfreezing stability and after-storage stability. The short-term temperature stability was assessed and compared after unfreezing the samples of two different concentrations mentioned above at room temperature

Figure 5. Calibration curve of OP1206 in plasma.

Table 2. Accuracy and precision results of OP 1206 analytical validation

Concentration (pg/mL)	Accuracy (CV %, n=5)		Precision (CV %, n=5)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.5	16.29	17.07	90.60	106.80
1.5	10.33	7.13	97.19	111.60
5.0	9.91	3.03	85.77	95.02
40.0	8.74	5.40	97.57	100.51

Table 3. Stability results of OP 1206 in human plasma

Criteria	Concentration (pg/mL)	Initial (%)	After storage (%)	
		Precision	Variation	Precision
Short term storage condition	1.5	0.39	-3.41	5.39
	40	1.78	-8.55	2.61
Repeating frozen condition	1.5	7.42	-9.35	5.64
	40	2.98	0.52	4.28
Solution in HPLC system condition	1.5	17.85	2.28	11.84
	40	0.95	-3.29	1.33

for 24 hours.

The freezing/unfreezing stability was assessed after repeating the process of freezing and unfreezing the samples of the two different concentrations more than three times. Samples of each concentration were stored at -70°C for 24 hours and were unfrozen at room temperature without any ancillary device. Under the same conditions, they were frozen again for 24 hours. After-storage stability was assessed after storing the samples of the two different concentrations within an auto-sampler (6°C) for 24 hours. The results of temperature stability assessment showed that the variation compared to the initial level was $-9.35\sim 0.52$ under every storage condition and that the value met the guidance for bioanalytical methods (Food and Drug Administration, 2018). The maximum precision was 17.85, which was within the allowable range (Table 3).

Conclusion

This study was carried out to identify the effects of the intake of evening primrose seed oil on improvement in the biosynthesis of prostaglandins. The highly-sensitive analysis method used in this study was optimized by applying the column switching system and improving recovery factors, and its specificity, accuracy, precision and linearity and the stability of samples were analyzed to validate the analysis method, showing allowable results. The highly sensitive analysis method using column switching effectively removed prostaglandins, endogenous substances, and OP 1206 was concentrated and analyzed by doing so. Since matrices were effectively removed through column switching, the liquid-liquid extraction method of which the recovery factor is relatively high could be used. Yield variances that might occur could be reduced by using internal standard. In this study, a highly-sensitive analysis method that could overcome the general quantitation limit of LC-MS/MS (10 pg/mL) was developed, and the developed analysis method is expected to be applied to actual bioavailability tests and to be utilized in testing the bioavailability, pharmacodynamics and bio-equivalence of new drugs made of natural substances.

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