Development and Validation of Oseltamivir Carboxylate and Oseltamivir Phosphate Quantification in Human Plasma by HPLC

นิพนธ์ต้นฉบับ

Original Article

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บทคัดย่อ

วัตถุประสงค์: เพื่อพัฒนาวิธีวิเคราะห์หาปริมาณโอเซลทามิเวียร์ คาร์บอกซิเลต (OC) และโอเซลทามิเวียร์ ฟอสเฟต (OP) ในพลาสมาของมนุษย์ โดยใช้วิธี HPLC อย่างง่าย **วิธีการศึกษา:** ในการแยกใช้คอลัมน์ชนิด Hypersil BDS cyano 5 µm (ความยาว 250 mm เส้นผ่านศูนย์กลาง 4.6 mm) ตรวจวัดโดย UV ที่ 230 nm ระบบเฟสเคลื่อนที่ คือ ระบบการปรับอัตราส่วน โดยเริ่มต้นที่ 50 มิลลิโมล ammonium acetate : acetonitrile (95:5) นาน 4 นาที และปรับความเข้มข้นเป็น 50 มิลลิโมล ammonium acetate: acetonitrile (70:30) 7 นาที ที่ 4 นาทีสุดท้าย ปรับเป็น 50 มิลลิโมล ammonium acetate: acetonitrile (95:5) **ผลการศึกษา:** ได้ค่าสัมประสิทธิ์การตัดสินใจของ OC และ OP เท่ากับ 0.9994 และ 0.9991 ตามลำดับ โดย OC และ OP แยกออกมาที่เวลา 4.35 และ 11.04 นาที ตามลำดับ ค่าปริมาณต่ำสุดที่สามารถวิเคราะห์ได้อย่างถูกต้อง (LOQ) เท่ากับ 1.5 µg/ml ค่าสัมประสิทธิ์ความแปรปรวนในระหว่างวันและภายในวันของ OC อยู่ในช่วง 2.15 - 7.19% และ 1.97 - 6.63% ตามลำดับ ส่วนของ OP พบในช่วง 2.41 - 8.52% และ 1.90 - 8.57% ตามลำดับ **สรุป:** ผลวิเคราะห์หาปริมาณโอเซลทามิเวียร์ คาร์บอกซิเลต และโอเซลทามิเวียร์ ฟอสเฟต ในพลาสมาของมนุษย์ โดยใช้วิธี HPLC ทำได้ง่ายและรวดเร็ว

คำสำคัญ: โอเซลทามิเวียร์ คาร์บอกซิเลต, โอเซลทามิเวียร์ ฟอสเฟต, HPLC, การตรวจสอบความถูกต้องของวิธีวิเคราะห์

Abstract

Objective: To develop a simple HPLC analysis for the quantification of oseltamivir carboxylate (OC) and oseltamivir phosphate (OP) in human plasma. **Method:** Chromatographic separation was carried out using Hypersil BDS cyano 5 µm column (length 250 mm, inner diameter 4.6 mm) with UV detection at 230 nm. The gradient system composed of 50 mM ammonium acetate: acetonitrile (95:5) for 4 min, followed by 50 mM ammonium acetate: acetonitrile (70:30) for 7 min, and finally 50 mM ammonium acetate: acetonitrile (95:5) for 4 min. **Results:** Coefficients of determination for the quantification of OC and OP were 0.9994 and 0.9991, respectively, with retention times of 4.35 and 11.04 min, respectively. The method limit of quantification for both compounds was 1.5 µg/ml. Coefficients of variation for the between- and within-day precision of OC were 2.15 - 7.19% and 1.97 - 6.63%, respectively, and those of OP were 2.41 - 8.52% and 1.90 - 8.57%, respectively. **Conclusion:** A simple and reliable HPLC quantification method of oseltamivir carboxylate and oseltamivir phosphate in human plasma was successfully validated.

Keywords: oseltamivir carboxylate, oseltamivir phosphate, HPLC, method validation

Introduction

Oseltamivir phosphate (OP) is an antiviral drug used for the treatment and prophylaxis of influenza virus A and influenza virus B.[1] OP is an ethyl ester pro-drug which is rapidly and extensively metabolized by esterases in the gastrointestinal tract and liver to its active form, oseltamivir carboxylate (OC) (Figure 1).[2,3]

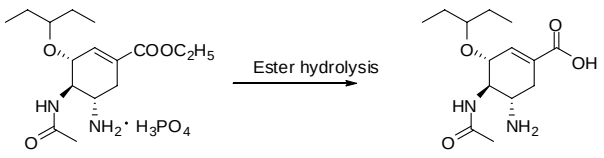

Figure 1 Oseltamivir phosphate, an ethyl ester pro-drug, rapidly and extensively metabolized by esterases in the gastrointestinal tract and liver to its active form, oseltamivir carboxylate.

Various analytical techniques have been explored to analyze OP and OC in the biological fluids and pharmaceutical preparations.[1,3-13] Green and co-workers reported the colorimetric method to approximate the amount of OP in capsules.[7] The HPLC-fluorescent detection was used to quantify OC in rat plasma by derivatizing OC with naphthalenedialdehyde; however, the method itself was toxic due to potassium cyanide (KCN) and required long incubation period for derivatization.[8] An enzymatic assay based on neuraminidase inhibition was also reported for the analysis of OC.[9] The HPLC methods with UV detection have been employed for the analysis of OC but not OP in human serum.[10,11] To date, there is no quantification method of both OC and OP in human serum by HPLC analysis.

Among the methods studied for the determination of OC and OP in various matrices to date, liquid chromatography is most commonly used. The LC-MS/MS method with solid phase extraction (SPE) was used to analyze rat and mouse brain samples with lower limit of quantification (LLOQ) of 60 ng/g for OP and OC.[3] Lindegårdh and coworkers also reported the LLOQ of 5 and 30 ng/ml for OP and OC, respectively, in urine by using respectively.[12] The present work reports the validation of a simple HPLC method for the quantification of both OP and its metabolite, OC, in human plasma.

Materials and Methods

Materials and reagents

OP was a generous gift from the Government Pharmaceutical Organization, Thailand. HPLC grade acetonitrile (J.T. Baker, USA) was used to prepare the mobile phase. Ultrapure water was obtained from a MilliQ Millipore system. Trichloroacetic acid and ammonium acetate were from Fluka, Buchs, Switzerland. Human plasma was kindly provided by the Thai Red Cross Society.

Instruments

The HPLC system was composed of a pump (model G13114) and autosampler connected with a Diode Array detector (model G1315A) (Agilent Technologies, Palo Alto, CA, USA). A class LC Chem Station G2170AA Data system (Agilent Spectral Module G2180AA) was used for data processing.

Synthesis of oseltamivir carboxylate (OC)

OP (0.41g, 1mmol) was dissolved in water (3 mL) and treated with triethylamine (1 mL). The mixture was stirred under nitrogen at room temperature overnight. The reaction mixture was then evaporated under reduced pressure and dried in high-vacuum oven overnight to obtain OC. The identity of OC was investigated by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectrometry.

Chromatographic conditions

Separation was accomplished using a BDS Hypersil Cyano column (length 250 mm, internal diameter 4.6 mm, particle size 5 µm), and with UV detection at 230 nm. The chromatographic separation was achieved using a gradient system (Table 1).

Table1 Gradient system used in chromatographic separation.

Time (min)	Ratio (50 mM ammonium acetate : acetonitrile)
0 – 4	95 : 5
5 - 12	70 : 30
13 - 17	95 : 5

Standard and sample preparation

Stock solutions of OC and OP at the concentration of 1 mg/ml were prepared in ultrapure water. Standard OC and OP solutions (1.5 - 50 µg/ml) were obtained by serial dilutions using ultrapure water.

The plasma was spiked with the OC and OP stock solutions to prepare the working samples at a concentration range of 1.5 - 50 µg/ml. Plasma (0.5 ml) was mixed with 20% w/v trichloroacetic acid (0.1 ml), vortexed for 10 min and centrifuged at 6,000 rpm for 5 min. The supernatant was collected and analyzed by HPLC.

Validation

Validation was performed following the FDA guidelines for bioanalytical methods.[14] The method was validated as regards its specificity, linearity, accuracy, precision (within- and between-day), sensitivity and stability. The stability of OC and OP samples was investigated after three consecutive freeze-thaw cycles.

To determine the accuracy of the method, three standard solutions with low, intermediate and high concentrations (1.5, 10, and 50 µg/ml, respectively) were analyzed. The percentage recovery was performed by six determinations and calculated by the relationship between the experimental concentration (C_{exp}) and the theoretical concentration (C_{theo}) expressed as percentage using the following equation: $100x (C_{exp}/ C_{theo})$.

To evaluate the within- and between-day precision, six replicates of three standard solutions at low, medium and high concentrations were assayed on the same day and six consecutive days, respectively.

Calibration

Linearity was studied at a concentration range of 1.5 - 50 µg/ml. The standard curve was analyzed by linear regression of peak height against OC and OP concentrations. Six concentrations of OC and OP standard solutions were carried out in six replicates on the same day. The correlation coefficient calibration and the regression analysis were

performed without applying any type of mathematical transformation or data weighing.

Results and Discussions

OC appeared as white foam with ^{1}H NMR (360 MHz, D_2O) δ 6.56 (t, *J*=2.1 Hz, 1H, C_2-*H*), 4.34 (d, *J*=9.0 Hz, 1H, C_3-*H*), 4.09 (dd, *J*=9.0, 11.7 Hz, 1H, C_4-*H*), 3.63-3.56 (m, 2H, C_5-*H* and 3-O*CH*$(Et)_2$), 2.95 and 2.53 (ABX, *J*=5.5, 11.8, 17.1 Hz, 2H, C_6-H_2), 2.15 (s, 3H, COCH_3), 1.67-1.49 (m, 4H, 2CH_2CH_3), 0.96 (t, *J*=7.4 Hz, 3H, CH_2CH_3), 0.91 (t, *J*=7.5 Hz, 3H, CH_2CH_3). ^{13}C NMR (100 MHz, D_2O) δ 175.56 (C), 174.23 (C), 133.51 (C), 132.84 (CH), 84.58 (CH), 76.10 (CH), 53.50 (CH), 50.04 (CH), 30.06 (CH_2), 25.87 (CH_2), 25.54 (CH_2), 22.73 (CH_3), 8.93 (CH_3), 8.85 (CH_3). *m/z* 285 $(M+H)^+$. These results confirmed identity of the compound as OC.

By using the developed sample preparation and validated chromatographic system, there were no interferences on the OC and OP peaks due to the components of the plasma samples. OC and OP were eluted at 4.35 and 11.04 min, respectively (Figure 2).

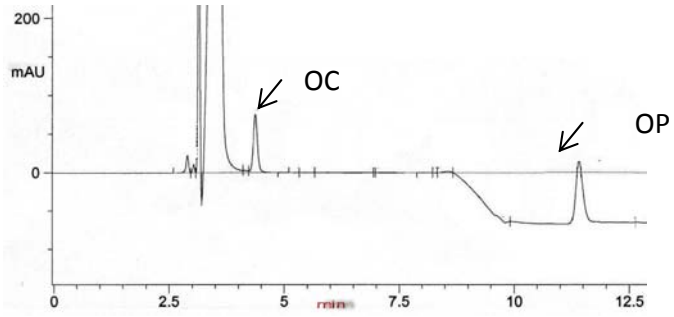

Figure 2 HPLC chromatogram showing the extract of plasma sample spiked with 50µg/ml OC and OP. Peak identifications at 4.35 and 11.04 min correspond to OC and OP respectively.

The recovery of the extraction of OC from human plasma at the concentrations of 1.5, 10 and 50 µg/ml ranged from 85.46 to 88.75%, and that of OP was from 87.23 to 90.67% (Table 2).

Table 2 Recoveries of OC and OP of the extracts.

Compound	% Recovery		
	1.5 µg/ml	10 µg/ml	50 µg/ml
OC	85.46	86.71	88.75
OP	87.23	87.25	90.67

The limits of quantification (LOQ) for OC and OP of the method were 1.5 µg/ml. Coefficients of correlation of the OC and OP calibration curves were greater than 0.99 (Figures 3 and 4). The results of the slope and ordinate values of the calibration curves were presented in Table 3.

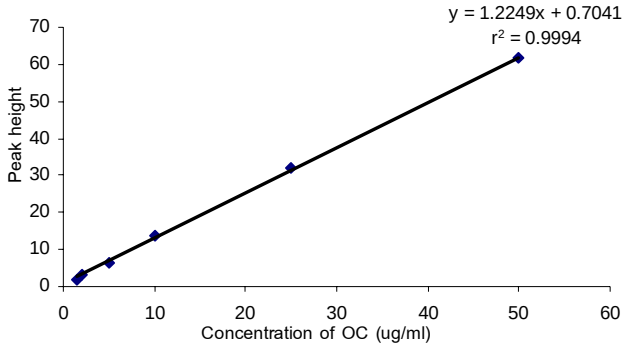

Figure 3 Linear relationship between peak heights and concentrations for the calibration range of OC (1.5 - 50 µg/ml).

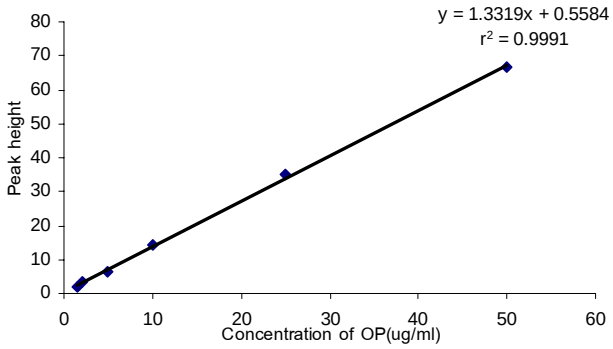

Figure 4 Linear relationship between peak heights and concentrations for the calibration range of OP (1.5 - 50 µg/ml).

Table 3 Calibration curve parameters from linear regression analysis.

Sample	Concentration µg/ml	Slope	Intercept	Coefficient of Determination (r^2)
OC	1.5-50	1.225	0.704	0.9994
OP	1.5-50	1.332	0.558	0.9991

The accuracy of the method was evaluated using three concentrations (low, intermediate and high) of the calibration at 1.5 - 50 µg/ml for OC and OP. Accuracy was measured using six determinations per concentration. The mean values were within 15% of the actual value except at LOQ, where it should not deviate by more than 20%.[14] The % recoveries of OC and OP ranged from 86.85 to 92.87% and 85.99 to

89.28%, respectively (Table 4). The accuracy results demonstrated that the results of the mean tests were close to the true concentrations of the analytes.

Table 4 Accuracy of OC and OP quantification.

Sample	Concentration (μg/ml)	% Recovery* (C_{exp}/ C_{theo})x100	S.D.
OC	1.5	86.85	0.13
	10	89.85	0.62
	50	92.87	1.23
	Mean ± CV	89.86	0.66
OP	1.5	85.99	0.15
	10	87.35	0.68
	50	89.28	1.43
	Mean ± CV	87.46	0.75

*Based on six determinations for each concentration.

The % coefficients of variation (CV) of the within-day precision for OC and OP were from 1.97 to 6.63% and 1.90 to 8.57%, respectively. The values of CV of the between-day precision for the OC and OP were between 2.15 - 7.19% and 2.41 - 8.52%, respectively (Table 5).

Table 5 Repeatability (within-day precision) and reproducibility (between-day precision) of the method.

Sample	Concentration (μg/ml)	Mean response ± S.D.	C.V. (%)
Within-day precision			
OC	1.5	1.81 ± 0.12	6.63
	10	12.24 ± 0.29	2.37
	50	57.90 ± 1.14	1.97
OP	1.5	1.75 ± 0.15	8.57
	10	12.88 ± 0.27	2.10
	50	60.49 ± 1.15	1.90
Between-day precision			
OC	1.5	1.78 ± 0.13	7.19
	10	12.21 ± 0.62	5.08
	50	57.16 ± 1.23	2.15
OP	1.5	1.76 ± 0.15	8.52
	10	12.56 ± 0.68	5.41
	50	59.41 ± 1.43	2.41

The effects of freeze-thaw cycle on the stability of OC and OP were evaluated at low, medium and high concentrations of the concentration range of 1.5 – 50 μg/ml. The tests were conducted at -20°C and -70°C as freezing temperature, and room temperature as thawing temperature. The sample recoveries were determined after three freeze-thaw cycles. The recoveries of OC and OP storing at -20°C were in the range between 87.37 - 92.68% and 83.47 - 87.16%, respectively (Table 6). On the other hand, those of OC and OP stored at -70 °C ranged between 82.98 - 92.20% and 89.39 - 93.01%, respectively. The results of stability test demonstrated that OC and OP were stable and no degradation was found due to the freeze-thaw process. OC and OP were stable in plasma during the assay period. The recovery of the stability of OC and OP were in a range of 82.98 - 93.01%, close to the determination of OC and OP in ferret and rat plasma reported by Wiltshire and co-workers (80.2 - 102.7%).[3]

Table 6 Freeze and thaw stability.

Sample	Concentration (μg/ml)	% Recovery (Mean ± S.D.) (Third-cycle)	
		-20 °C	-70 °C
OC	1.5	87.37 ± 0.18	82.98 ± 0.09
	10	92.68 ± 0.54	88.58 ± 0.34
	50	89.29 ± 1.43	92.20 ± 1.75
OP	1.5	83.47 ± 0.24	90.68 ± 0.05
	10	85.48 ± 0.49	89.39 ± 0.74
	50	87.16 ± 1.18	93.01 ± 1.41

The results proved that a simple HPLC method was successfully validated. The method was reliable for the quantification of OC and OP in human plasma. Because solid phase extraction, commonly used in various studies, is relatively expensive, the sample preparation by acid precipitation in this study benefits the reduction of analysis cost. As OC and OP are highly soluble in water, trichloroacetic acid is an appropriate choice for protein precipitation. The results also proved that no matrix effects were detected. The validated HPLC method used low amount of organic solvent; as a result, it had not only low direct environmental toxicity but also low risk to the health of analyst.

The LOD of 0.95 μg/ml for OC and OP in this study was higher than the LLOQ of 1 ng/ml for OP and OC in human plasma and urine using LC-tandem MS[13] and the LOQ of 0.05 μg/ml for OC in rat plasma using fluorescent detection.[8] However, HPLC/fluorescence method by pre-column derivatization with naphthalenedialdehyde was not available for a large number of plasma samples because the method required long incubation period and the use of KCN as the derivatizing agent can cause toxicity.[8] It is commonly

accepted that LC-MS-MS facilitates the injection of low sample volume and MS-MS detection is more sensitive than UV and fluorescent detection; nevertheless, the cost of instrument hinders the implementation of the technique in some laboratories. The high LOQ values for the determination of OC and OP achieved in this study were due to the fact that the old generation of diode array detector was employed. It is commonly known that the diode array detector is less sensitive than the normal UV-VIS detector. Therefore, the use of UV-VIS detector will benefit lower LOQ values of both OC and OP. This study is a prototype of the use of a simple and reliable HPLC method for the determination of OC and OP in human plasma.

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