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A Sensitive, Efficient, and Cost-Effective Method to Determine Rotigotine in Rat Plasma Using Liquid-Liquid Extraction (LLE) and LC-MRM

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Abstract : Rotigotine (RTG) is a non-ergot dopamine agonist used to manage the early stage of Parkinson's disease (PD) as transdermal patch. However, the poor medication compliance of PD patients and skin issues related with repeated applications of RTG patches lead to the search for alternative formulations and it also requires appropriate analytical methods for their *in vivo* evaluation. Thus, here, a sensitive, efficient, and cost-effective method to determine RTG in rat plasma using liquid-liquid extraction (LLE) and multiple reaction monitoring was developed. The use of 20 μL of rat plasma for sample treatment, 8-OH-DPAT as the internal standard, and methyl *tert*-butyl ether as the LLE solvent in the present method gives it advantages over previous methods for the analysis of RTG in biological samples. The good analytical performance of the developed method was confirmed in specificity, linearity (the coefficient of determination ≥0.999 within 0.1-100 ng/mL), sensitivity (the lower limit of quantitation at 0.1 ng/mL), accuracy (81.00–115.05%), precision (≤10.75%), and recovery (81.00-104.48%) by following the FDA guidelines. Finally, the applicability test of the validated method to the *in vivo* evaluation of a RTG formulation showed that the present method is the only method which can be accurately applied to that longer than 24 hours, critical for the development of formulations with reduced dosing frequencies. Therefore, the present method could contribute to the development of new RTG formulations helpful to people suffering from PD.

Keywords : rotigotine, multiple reaction monitoring, liquid-liquid extraction, rat plasma, pharmacokinetics

Introduction

Rotigotine (RTG, Figure 1A) is a non-ergot dopamine agonist used to manage the early stage of Parkinson's disease (PD).[1] Due to its poor oral bioavailability (1%) by the extensive first-pass hepatic clearance and nonpolar characteristics (logP of 5.17), its formulation in the market is limited to transdermal patch which shows advantages like the relatively long dosing interval (once a day) and the reduction of motor adverse effects including dyskinesia, motor fluctuations, and resting tremor.[2] However, the poor medication compliance of PD patients and skin issues, such as erythema, pruritus, and dermatitis, related with repeated applications of RTG patches lead to the search for alternative formulations with reduced dosing frequencies.[3] Thus, to facilitate the development of new RTG formulations, appropriate analytical methods for their *in vivo* evaluation are needed.

Recently, liquid chromatography and multiple reaction monitoring assay (LC-MRM), a considerably specific and sensitive technique which belongs to liquid chromatography and tandem mass spectrometry (LC-MS/MS) is commonly chosen for drug analyses and it has been widely used for the *in vivo* evaluation of RTG formulations, too.[4-6] In the case of sample treatment, also important due to its preventive effect to signal suppression among co-eluting compounds from an LC column in LC-MS/MS, protein precipitation and liquid-liquid extraction (LLE) have been generally employed for the determination of RTG in biological samples.[7-9] However, protein precipitation, mainly removing proteins from a sample, may not be effective to solve the suppression effect of nonpolar analytes like RTG to keep the quantitative property of the method.[10] Until now, Sha *et al.*'s method to determine RTG in rat plasma using LLE and LC-MRM

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seems to be the most acceptable in the community, there are still some margins to be improved.[8] First, while its lower limit of quantitation (LLOQ, 0.2 ng/mL) is lower than others, it may not be sensitive enough for longer-period pharmacokinetic studies.[8] Also, the demand of relatively large volume of plasma (50 μL) and high cost brought by the use of a stable isotope RTG (RTG-d_3) as an internal standard (IS) are additional drawbacks.[8]

Thus, here, a sensitive, efficient, and cost-effective method to determine RTG in rat plasma using LLE and LC-MRM was developed. The use of 20 μL of rat plasma for sample treatment, 8-OH-DPAT (CAS number: 78950-78-4) as the IS, and methyl *tert*-butyl ether (MTBE) as the LLE solvent in the present method gives it advantages over previous methods for the analysis of RTG in biological samples. The developed method was validated in various parameters according to FDA guidelines and its applicability to longer-period pharmacokinetic studies was also confirmed.

Experimental

Chemicals and reagents

RTG (≥99.0%), 8-OH-DPAT (≥99.0%) used as the IS, ammonium formate (LC-MS grade), and formic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile for HPLC, MTBE, and water were obtained from J. T. Baker (Phillipsburg, NJ, USA).

Preparation of standard solutions

To prepare stock solutions, RTG and the IS were both dissolved at 1 mg/mL in acetonitrile. The RTG stock solution was diluted with acetonitrile to 160 ng/mL (the RTG working solution), and the extraction solvent was prepared by the dilution of the IS stock solution with MTBE to 200 ng/mL. All stock solutions and working solutions including the extraction solvent were stored at -27°C, until use.

Liquid-liquid extraction (LLE)

An aliquot (20 mL) of rat plasma was mixed with 500 μL of the extraction solvent including the IS using a vortex mixer for a minute. After centrifugation of the mixture at 12,000 × g for 10 minutes, the whole top layer (the extraction solvent layer) was transferred to a micro-centrifuge tube. Then, the solution taken was dried at room temperature under nitrogen stream, and the resulting residue was reconstituted in 100 L of acetonitrile. The final solution was centrifuged at 12,000 × g for 10 minutes, and a part of its supernatant was analyzed by LC-MS/MS. A matrix-matched standard (MMS) and a standard-spiked sample (SSS) were prepared by adding an appropriate volume of the RTG working solution into the final LLE extract obtained from blank rat plasma and into blank rat plasma prior to the LLE steps, respectively. For the present study, SSSs were employed as QC samples (0.1, 0.3, 40, and 80 ng/mL for LLOQ, low QC (LQC), middle QC (MQC) and high QC (HQC), respectively). Also, MMSs (0.1, 2, 10, 25, 50, and 100 ng/mL) were used for building calibration curves.

Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

For LC-MRM, a Shimadzu Nexera UPLC system (Tokyo, Japan) and a Shimadzu LCMS 8060 triple quadrupole mass spectrometer were interfaced through electrospray ionization (ESI) in positive ion mode. For LC separation, a Waters Atlantis HILIC Silica column (2.1 × 150 mm, 3 mm, Santa Clara, CA, USA) and the isocratic mobile phase condition (the volumetric ratio of 2 mmol/L of an aqueous ammonium formate solution including 0.1% (v/v) formic acid (MP A) to acetonitrile including 0.1% (v/v) formic acid (MP B), 15:85) were used. A sample was separated at the flow rate of 0.25 mL/min for eight minutes, and the autosampler and the column oven were kept at 4 and 40°C, respectively. For ESI, source parameters were set as follows: nebulizing gas flow at 2 L/min, heating gas flow at 10 L/min, drying gas flow at 10 L/min, interface temperature at 300°C, DL temperature at 250°C, and heating block temperature at 400°C. In the case of MRM, three MRM transitions per compound were monitored: one with the highest sensitivity was the screening transition used for the quantitation and the others were the confirmatory transitions for the target identity confirmation. In the case of RTG, 316.1 *m/z* (precursor ion) / 147.3 *m/z* (product ion)/ -24 V (collision energy), 316.1 *m/z* / 77.1 *m/z* / -73 V, and 316.1 *m/z* / 107.1 *m/z* / -64 V were the screening transition, the confirmatory transition 1, and the confirmatory transition 2, respectively. In addition, the screening transition of 248.1 *m/z* / 147.1 *m/z* / -23 V, the confirmatory transition 1 of 248.1 *m/z* / 91.2 *m/z* / -42 V, and the confirmatory transition 2 of 248.1 *m/z* / 102.1 *m/z* / -17 V were applied for the IS. All mass spectrometry data were acquired and analyzed using Lab Solutions (version 5.93, Shimadzu). For quantitation, three pre-requirements (all three transition peaks should have the same retention time; the signal to noise ratio (S/N) of the screening transition peak should be higher than 10; all confirmatory transition peaks should have the S/N values higher than 3) were checked. When all they were satisfied, a screening transition peak area ratio of RTG to the IS was calculated and used for quantitation.

Application to pharmacokinetic study in rats

The validated LC-MS/MS method was employed to determine the plasma concentration-time profile of RTG, following topical application of a RTG microemulsion formula. The animal study was carried out after the approval of the Institutional Animal Care and Use Committee (IACUC) of Dankook University (DKU-22-

045, Cheonan, South Korea). Seven-week-old male Sprague-Dawley rats (200 ± 20 g) acquired from Samtako Bio Korea (Gyeonggi-do, South Korea) were kept under controlled environmental conditions (23 ± 1°C, 12 h day/ 12 h night) with free access to standard food and water. After 3 days of acclimatization period, the hair in the dorsal region was shaved and then RTG-loaded microemulsion hydrogel consisted of 2% (w/v) RTG was topically administered at 2 mg/kg as RTG.[11] At predetermined time, the rat blood samples (approximately 0.2 mL) were collected in heparinized 1.5 mL polythene tubes from the jugular vein. The collected blood was centrifuged at 13,000 rpm for 10 minutes and the resulting plasma was taken and kept in a deep freezer at -80°C until its treatment using LLE. Pharmacokinetic parameters of RTG, such as the maximum drug concentration in plasma (C_{max}), time to reach C_{max} (T_{max}), area under the curve for drug concentration in plasma-time (AUC), and elimination half-life ($T_{1/2}$), were calculated from the pharmacokinetic profile, using a WinNonlin® version 5.2 program (Pharsight Co., Mountain View, CA).

Results and discussion

Method development

Liquid chromatography and multiple reaction monitoring

For the present study, 8-OH-DPAT (Figure 1B), whose chemical structure is similar with that of RTG, was selected as the IS. Since 8-OH-DPAT is much cheaper than stable isotope –labeled RTG s such as RTG-d_3, the present method has cost advantage.[8] $[M+H]^+$ ions (316.1 *m/z* and 248.1*m/z* for RTG and the IS, respectively) were chosen as precursor ions. Product ions for MRM were chosen from product ion scan (PIS) results of individual precursor ions. The strongest fragment ions (147.3 *m/z* and 147.1 *m/z* for RTG and the IS, respectively) were chosen for quantitation. As confirmatory transitions for identity confirmation, the second and third strongest intensities (77.1 and 107.1 *m/z* for RTG and 91.2 and 102.1 *m/z* for the IS) were selected. In the case of separation, a HILIC silica column and the isocratic mobile phase condition (the volumetric ratio of MP A to MP B, 15:85) were used for the efficient separation of components including RTG and the IS with less suppression effect within eight minutes. While RTG is non-polar, it is a base due to its tertiary amine bound to three electron donating groups. It means that if the pH of its solution is kept much lower than the pKa value of its mono-protonated conjugate acid (about 10), it mainly exists as its conjugate acid form whose relative polarity is higher than that of its free from. Thus, at the mobile phase condition of the present method (about pH 3), majority of RTGs are kept as mono-protonated RTG cations which may have attraction with deprotonated free silanol groups at the surface of silica packing materials as well as retainability in polar liquid stationary phase film of the HILIC silica column.

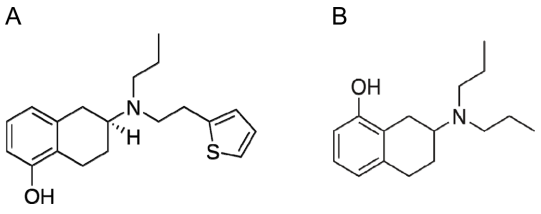

Figure 1. Chemical structures of rotigotine (A) and 8-OH-DPAT (B)

Sample preparation

For the development of highly sensitive and simple sample preparation steps which can be applied to rat plasma, LLE was chosen in the present study.[10] Some organic solvents including MTBE, ethyl ether, and their mixtures were compared to find the optimal extraction solvent for RTG. Since MTBE showed much higher recovery of RTG (103.89 ± 4.13%, n=3) from a SSS (0.1 ng/mL of RTG) than others (63.57 ± 6.82, 68.72 ± 6.56, 75.44 ± 6.23, and 82.22 ± 5.39% from ethyl ether, the mixture of 70% v/v of ethyl ether and 30% v/v MTBE, the mixture of 50% v/v of ethyl ether and 50% v/v MTBE, and the mixture of 30% v/v of ethyl ether and 70% v/v MTBE, respectively, n=3), MTBE was selected as the LLE solvent in the present study. The volume of rat plasma required for the sample preparation was decided to 20 mL, the minimal volume which showed precise and linear results from comparison experiments of various plasma volumes (data not shown). To the best of our knowledge, the present sample preparation method is the most efficient one in the aspect of the volume of rat plasma demanded to determine RTG in it (20 mL in the present method *vs*. 50 mL in Sha *et al.*'s method, the previously most efficient one) and it may be explained by the better extraction of RTG by MTBE.[8] Also, the deposit of contaminants originated from rat plasma on the curtain plate of the mass spectrometer by continuous analyses of prepared samples was checked and there was not any significant sign of contamination in the system.

Method validation

The present method was validated in specificity, linearity, sensitivity, accuracy, precision, and recovery according to the FDA guidelines.[12] First, the specificity of this method was confirmed by comparison between blank rat plasma and the LLOQ sample (Figure 2). In the chromatogram from the LLOQ sample, RTG and the IS peaks were identified at about 4.8 and 5.1 minutes, respectively, but both were not observed from the blank plasma analyses. Also, the good linearity (the coefficient of determination, $r^2 \geq 0.998$) of the method was confirmed over the concentration range between 0.1 and 100 ng/mL, (n=6, Table 1). Third,

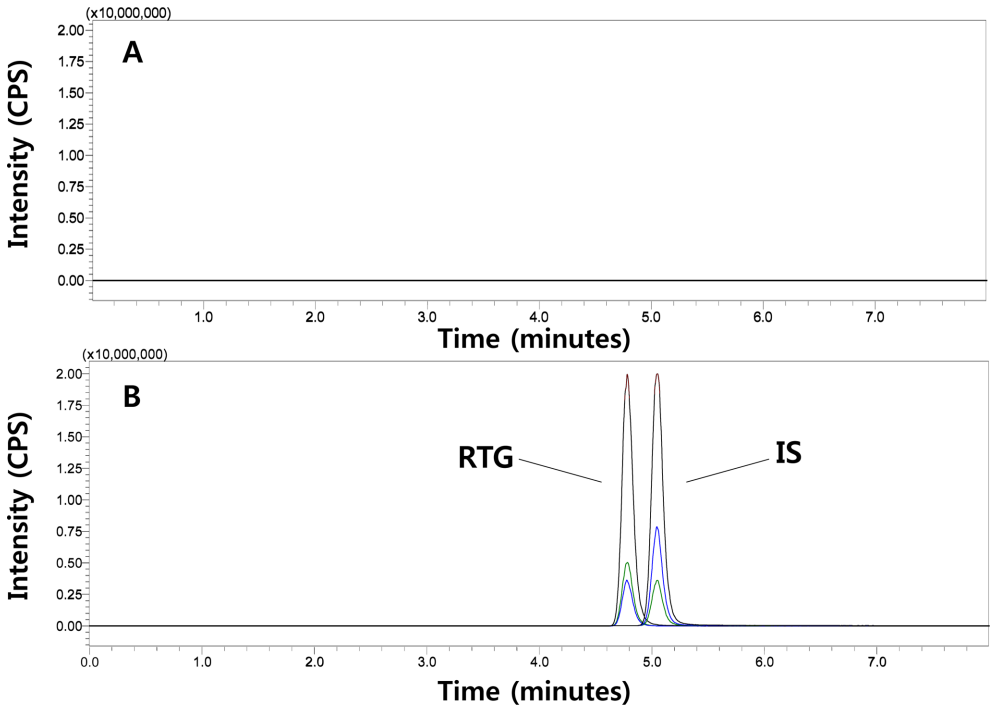

Figure 2. Multiple reaction monitoring chromatograms of blank rat plasma (A) and rat plasma including 50 ng/mL of RTG and IS (B). RTG and IS stand for rotigotine and 8-OH-DPAT, respectively.

accuracy and precision estimated from all QC sample results were good enough to satisfy the criteria of FDA guidelines: the intra-day accuracy between 83.48 and 115.05%; the inter-day accuracy between 81.00 and 113.50%; the intra-day precision, not more than 7.43%; inter-day precision, not more than 10.75% (Table 2). Finally, good recovery (the percentage of the RTG screening transition peak area of a QC sample to that of its counter MMS with the same RTG concentration) between 81.00 and 104.48% was observed (Table 2). Based on all validation results, LLOQ, the lowest concentration showing good accuracy, precision, and recovery within the linear dynamic range was confirmed as 0.1 ng/mL and it is proven to be the most sensitive method to determine RTG in rat plasma (0.1 ng/mL in the present method *vs.* 0.2 ng/mL in Sha *et al.*'s method, the previously most sensitive one).[8] The more improved sensitivity of the present method than those of previous ones may be

Table 1. Information from the calibration curves of rotigotine in rat plasma (n = 6)

Concentration range (ng/mL)	Slope	y-Intercept	R^2
	Mean ± SD	Mean ± SD	
0.1 - 100	0.0247 ± 0.0019	0.0003 ± 0.0002	≥ 0.999

Table 2. Accuracy, precision, and recovery confirmed by LC-MRM analyses of rotigotine (RTG) in rat plasma (n = 6)

Types	Nominal concentration of RTG (ng/mL)	Calculated concentration of RTG (ng/mL)	Accuracy (%)	Precision (%)	Recovery (mean ± standard deviation, %)
Intra-day	0.1	0.11±0.01	111.91	4.20	98.45 ± 4.50
	0.3	0.28±0.02	93.89	7.43	93.65 ± 7.98
	40	34.34±0.85	85.84	2.11	85.53 ± 2.11
	80	74.10±2.62	92.63	3.27	95.53 ± 3.37
Inter-day	0.1	0.11±0.01	112.56	10.75	96.09 ± 4.13
	0.3	0.28±0.02	93.73	7.13	93.84 ± 6.04
	40	34.57±1.11	86.42	2.78	85.43 ± 2.72
	80	71.78±3.13	89.73	3.91	91.76 ± 4.31

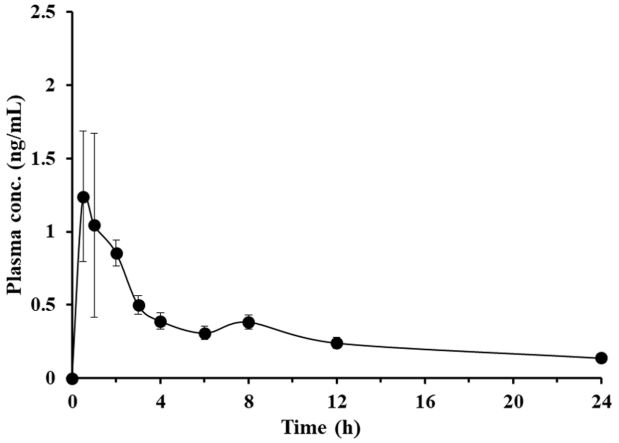

Figure 3. Plasma concentration-time profile of rotigotine (RTG) following topical application of RTG loaded microemulsion hydrogel in normal rats (2 mg/kg as RTG). Data represent mean ± standard deviation (n = 4).

Table 3. Pharmacokinetic parameters of rotigotine (RTG) following topical application of RTG -loaded microemulsion hydrogel in normal rats (2 mg/kg as RTG)

Parameter	Mean ± Standard deviation (n=4)
$AUC_{(0\text{-}24\ h)}$ (ng·h/mL)	7.60 ± 1.43
$AUC_{(0\text{-}\infty)}$ (ng·h/mL)	10.84 ± 3.63
C_{max} (ng/mL)	1.96 ± 0.78
T_{max} (h)	1.00 ± 0.71
$T_{1/2}$ (h)	9.69 ± 4.51

explained by the better extraction of RTG by MTBE, the LLE solvent.

Pharmacokinetic profile of RTG following topical application in rats

The plasma concentration-time profile of RTG after topical application is depicted in Figure 3. Additionally, the relevant pharmacokinetic parameters such as AUC, C_{max}, T_{max}, and elimination $T_{1/2}$ calculated from the pharmacokinetic profiles are represented in Table 3. After topical administration, the level of RTG in plasma increased rapidly and reached C_{max} (1.96 ng/mL) after an hour (T_{max}). Afterward, the drug concentration in plasma gradually decreased over 24 hours, with the extended elimination $T_{1/2}$ of 9.98 ± 3.95 hours. The $AUC_{(0\text{-}24\ h)}$ and $AUC_{(0\text{-}\infty)}$ values of RTG, an indicator of the extent of drug absorption, were determined to 7.60 and 10.84 ng·h/mL, respectively. The plasma concentration of RTG after 24 hours of post-dosing was determined to 0.137 ng/mL, which was about 1.37-fold higher than LLOQ (0.1 ng/mL) of the currently established method. It strongly suggests that the present method with LLOQ at 0.1 ng/mL is the only method which can be accurately applied to the *in vivo* evaluation of RTG formulation longer than 24 hours, critical for the development of formulations with reduced dosing frequencies.[3]

Conclusions

A sensitive, efficient, and cost-effective method to determine RTG in rat plasma using LLE and MRM was developed. The use of 20 μL of rat plasma for sample treatment, 8-OH-DPAT as the IS, and MTBE as the LLE solvent in the present method gives it advantages over previous methods for the analysis of RTG in biological samples. The developed method was validated in various parameters including specificity, linearity, sensitivity, accuracy, precision, and recovery by following the FDA guidelines. Finally, the applicability test of the validated method to the *in vivo* evaluation of a RTG formulation showed that the present method with LLOQ at 0.1 ng/mL is the only method which can be accurately applied to that longer than 24 hours, critical for the development of formulations with reduced dosing frequencies. Since the present study resulted the most sensitive as well as efficient method to determine RTG in rat plasma, it could contribute to the development of new RTG formulations helpful to people suffering from PD.

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