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A Simple and Efficient Method to Determine Rivaroxaban in Rat Plasma Using Liquid-Liquid Extraction and LC-MRM

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Abstract : Rivaroxaban (RRN) is the first available active direct factor Xa inhibitor (anticoagulant) with oral administration. Due to its success in market, there have been efforts to develop various RRN formulations, and the development of good analytical methods for its *in vivo* evaluation is an essential prerequisite. Thus, here, a simple and efficient method to determine RRN in rat plasma using liquid-liquid extraction (LLE) and liquid chromatography and multiple reaction monitoring (LC-MRM) was presented. The use of ethyl acetate as the LLE solvent results appropriate extraction and purification of RRN and it also helps the significant reduction of rat plasma volume required for RRN quantitation. The developed method showed good analytical performance including specificity, linearity ($r^2 \geq 0.999$ within 0.5 - 500 ng/mL), sensitivity (the lower limit of quantitation at 0.5 ng/mL), accuracy (89.3 - 107.0%), precision (≥ 12.7%), and recovery (89.2 - 105.7%). Additionally, RRN in sample extracts showed good stability. Finally, the applicability of the validated method to the PK evaluation of RRN was confirmed after its oral administration to normal rats. The present method is the first analytical method employing LLE for the simple and efficient extraction and purification of RRN in rat plasma. Therefore, the present method can contribute to the development of new RRN formulations as well as to the monitoring of RRN in special clinical situations through its efficient determination in various samples with or without minor modification.

Keywords : Rivaroxaban, Multiple reaction monitoring, Liquid-liquid extraction, Rat plasma, Pharmacokinetics

Introduction

Rivaroxaban (RRN) is the first available active direct factor Xa inhibitor (anticoagulant) with oral administration.[1-3] Due to its more predictable pharmacokinetics and pharmacodynamics and less drug-drug and food-drug interaction cases than those of coumarin-related anticoagulants, the number of its administration to patients have been increased.[4-6] Thus, interests on the development of various RRN formulations and new drugs with similar chemical structures and/or pharmacological functions have also increased, and, as a result, the need of the analytical method for their in vivo evaluation have existed. Additionally, the monitoring of RRN in special situations including acute renal failure and stroke increases the demand of reliable and sensitive methods to determine RRN.[7-9] Recently, liquid chromatography and multiple reaction monitoring assay (LC-MRM), a substantially specific and sensitive technique generally employed for drug analyses, has been widely used for these purposes.[10-13] However, their sample preparation methods are too simple to remove matrix components effectively (protein precipitation)[10,12,13] or too complicated to complete sample preparation quickly (solid phase extraction, SPE).[11]

Thus, here, a simple and efficient method to determine RRN in rat plasma using liquid-liquid extraction (LLE) and LC-MRM is presented. The developed method was validated in various parameters including specificity, linearity, sensitivity, accuracy, precision, recovery, and stability. The validated method was successfully applied to the PK evaluation of RRN after its oral administration to normal rats at a dose of 0.5 mg/kg.

Experimental

Chemicals and reagents

RRN (Fig. 1A, 99.9%) and apixaban (Fig. 1B, 99.6%) used as the internal standard (IS) were provided by Alembic Pharmaceuticals (Gujarat, India) and Honour Lab

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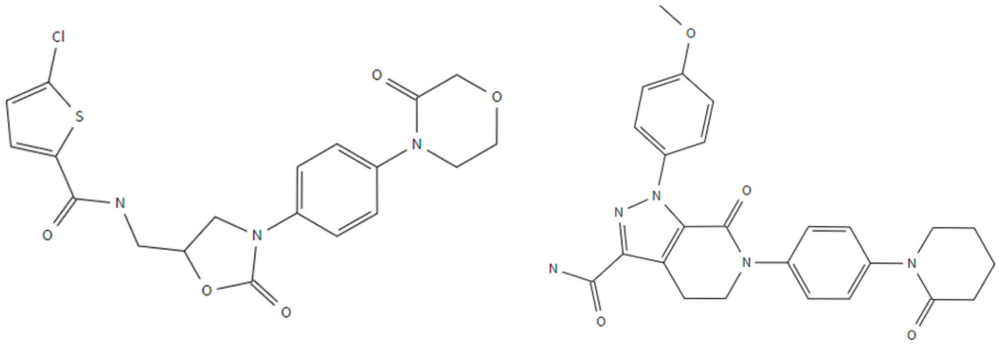

A. Rivaroxaban ($C_{19}H_{18}ClN_3O_5S$) B. Apixaban ($C_{25}H_{25}N_5O_4$)

Figure 1. Chemical structures of rivaroxaban (A) and apixaban (B).

(Telangana, India), respectively. Formic acid and dimethyl sulfixude (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile for HPLC and ethyl acetate were supplied by Burdick & Jackson (Muskegon, MI, USA) and J.T. Baker (Phillipsburg, NJ, USA), respectively. Ultrapure water was produced by a Millipore Milli-Q system (Bedford, MA, USA).

Preparation of calibration and quality control samples

RRN and IS were dissolved in DMSO at a concentration of 1 mg/mL to prepare stock solutions. The RRN stock solution was diluted with acetonitrile to 500 ng/mL (the RRN working solution). In the case of IS, its working solution was prepared by the dilution of its stock solution with ethyl acetate to 14 ng/mL. All stock solutions and working solutions were stored at -80°C and 4°C, respectively, until use. Calibration and quality control (QC) samples were prepared by spiking the RRN working solution to blank plasma sampled from male Sprague–Dawley rats (Orient Bio, Seongnam, South Korea) weighing approximately 300 g for a specific concentration of RRN. In the present study, a total of six calibration samples (0.5, 10, 50, 100, 250, and 500 ng/mL) and four QC samples (0.5, 1.5, 200, and 400 ng/mL for lower limit of quantification (LLOQ), low QC (LQC), middle QC (MQC) and high QC (HQC), respectively) were prepared.

Liquid-liquid extraction

An aliquot (20 mL) of a rat plasma sample, a calibration sample, or a quality control sample was mixed with 500 mL of the IS working solution using a vortex mixer for a minute. Ethyl acetate, the solvent of the IS working solution took the role of the LLE solvent. After centrifugation of the mixture at 13,500 × g for 10 minutes, the whole supernatant was transferred to a micro-centrifuge tube. Then, the supernatant was dried at 40°C under nitrogen stream, and the resulting residue was reconstituted in 100 mL of a 30% (v/v) aqueous acetonitrile solution under ultrasonication for three minutes. The final solution was centrifuged at 13,500 × g for 10 minutes, and a part of its supernatant was analyzed by LC-MRM.

Liquid chromatography and multiple reaction monitoring

For LC-MRM, a Shimadzu Nexera UPLC system (Tokyo, Japan) and a Shimadzu LCMS 8050 triple quadrupole mass spectrometer (Tokyo, Japan) were interfaced through electrospray ionization (ESI) in positive ion mode. Also, a Phenomenex Luna C18 column (2.0 × 150 mm, 5 mm) and the isocratic mobile phase (MP) condition of a 45% (v/v) aqueous acetonitrile solution including 0.1% (v/v) formic acid were used for LC. The total separation time was five minutes and the flow rate of MP was 0.25 mL/min. The column and the autosampler were kept at 40°C and 4°C, respectively. For ESI, source parameters were set as follows: nebulizing gas flow at 3 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 transitions per target compound were employed; one with higher sensitivity was used for the purpose of quantitation and the others were used for the confirmation of the compound identity. In the case of RRN, 436.1 *m/z* (precursor ion) / 145.0 *m/z* (product ion) / -27 V (collision energy), 436.1 *m/z* / 73.0 *m/z* / -87 V, and 436.1 *m/z* / 231.0 *m/z* / -22 V were used for quantitation (screening transition), confirmation (confirmatory transition 1), and extra confirmation (confirmatory transition 2), respectively. Additionally, the screening transition of 460.1 *m/z* / 443.2 *m/z* / -24.5 V, the confirmatory transition 1 of 460.1 *m/z* / 119.1 *m/z* / -39.4 V, and the confirmatory transition 2 of 460.1 *m/z* / 282.0 *m/z* / -35.4 V were applied for IS. All mass spectrometry data were acquired and analyzed using Lab Solutions (version 5.93, Shimadzu). In particular, a screening transition peak area ratio of RRN to IS was calculated for quantitation purposes. The requirements for quantitation are 1) all three transition peaks should have the same retention time, 2) the signal to noise ratio (S/N)

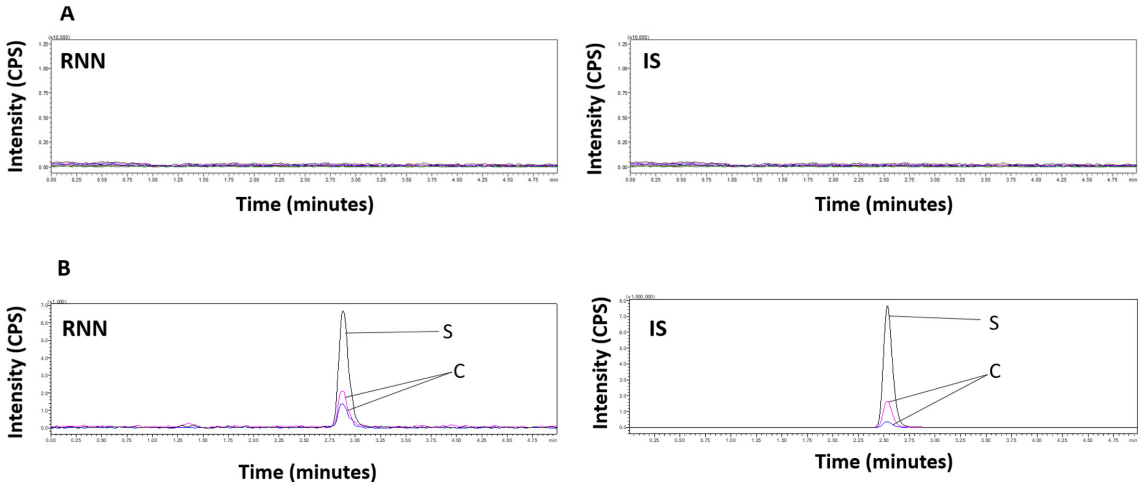

Figure 2. Multiple reaction monitoring chromatograms of rivaroxaban (RRN) and apixaban (IS) in rat plasma. Blank plasma (A) and plasma including 0.5 ng/mL of RRN and 14 ng/mL of IS (B). "S" and "C" stand for the screening transition peak and the confirmatory transition peaks, respectively.

of the screening transition peak should be higher than 10, and 3) all confirmatory transition peaks should have the S/N values higher than 3.

Results and Discussion

Method development

Liquid chromatography and multiple reaction monitoring. In this LC-MRM study, singly-protonated cations (436.1 *m/z* and 460.1 *m/z* for RRN and IS, respectively, supplementary information) were monitored as precursor ions. Product ions for MRM were chosen based on ion intensities observed in individual product ion scan (PIS) studies of precursor ions. Fragment ions with the strongest intensities in PIS spectra (145.0 *m/z* and 443.2 *m/z* for RRN and IS, respectively, supplementary information) were selected for the purpose of quantitation (screening transition) and PIS fragment ions with the second and third strongest intensities (73.0 and 231.0 *m/z* for RRN and 119.1 and 282.0 *m/z* for IS, supplementary information) were picked for the purpose of confirmation (confirmatory transition). Additionally, a C18 column and an isocratic mobile phase condition (45:55 of 0.1% formic acid in water:0.1% formic acid in acetonitrile, v/v) were used in this method for the efficient separation of components of a sample, and, as a result, RRN and IS were separated and detected within five minutes.

Sample preparation. In order to develop a simple and efficient sample preparation method, methods based on chromatography such as SPE and QuEChERS were excluded in the present study. Thus, only LLE was considered as the sample preparation method, and some organic solvents such as ethyl acetate, methyl *tert*-butyl ether, and ethyl ether were compared to find the optimal extraction solvent for RRN. As a result, ethyl acetate, which resulted in the best recovery (105.7%) of RRN from RRN-spiked (1.5 ng/mL) rat plasma was chosen as the LLE solvent in the present study (data not shown). The volume of rat plasma, 20 L, used in this method was decided by comparisons of results from experiments started from various volume of rat plasma. It was the minimal volume which produced data precise and linear enough for quantitation (data not shown). Since at least 200 mL of plasma was required for previous RRN studies, the present method has an advantage of consuming more than 10-times less volume of sample.[10-13] Also, the contamination by continuous analyses of prepared samples was checked and there was not any significant sign of contamination in the system and data.

Method validation

The developed method was validated in terms of specificity, linearity, sensitivity, accuracy, precision, recovery, and stability according to the FDA guidelines for validation of bioanalytical methods (supplementary information).[14] The specificity of this method was determined by comparison between blank rat plasma with the LLOQ sample. In the case of the LLOQ sample, RRN and IS peaks were confirmed at about 2.9 and 2.6 minutes, respectively, but they were not observed from blank rat plasma analyses (Fig. 2). The linearity ($r^2 \geq 0.999$) was observed within the concentration range between 0.5 and 500 ng/mL, and the lowest concentration of the linear concentration range, 0.5 ng/mL was confirmed as the LLOQ of this method. From the analyses of QC samples, accuracy and precision of this method were evaluated, and values for all related parameters are as below: intra-day accuracy range, inter-day accuracy range, intra-day

Table 1. Intra- and inter-day accuracy and precision from LC-MRM of rivaroxaban (RRN) in rat plasma (n = 6).

Types	Nominal concentration of RRN (ng/mL)	Calculated concentration of RRN (ng/mL)	Accuracy (%)	Precision (%)
Intra-day	0.5	0.47 ± 0.03	93.1	6.9
	1.5	1.52 ± 0.16	101.3	10.7
	200	213.98 ± 2.50	107.0	1.2
	400	420.31 ± 8.79	105.0	2.1
Inter-day	0.5	0.45 ± 0.03	89.3	6.1
	1.5	1.42 ± 0.18	94.7	12.7
	200	200.93 ± 11.27	100.5	5.6
	400	413.66 ± 12.34	103.4	3.0

Table 2. Recovery of rivaroxaban (RRN) in rat plasma.

RRN concentration (ng/mL)	Mean recovery (standard deviation)
0.5	95.2 (5.8)
1.5	105.7 (10.9)
200	89.2 (1.0)
400	93.1 (1.9)

Table 4. Pharmacokinetic parameters of rivaroxaban (RRN) following oral administration of a RRN raw material at a dose of 0.5 mg/kg in rats (mean ± standard deviation, n = 6).

Parameters	A RRN raw material
$AUC_{0\text{-}24h}$ (ng·h/mL)	364.17 ± 99.96
$AUC_{0\text{-}\infty}$ (ng·h/mL)	382.75 ± 90.20
C_{max} (ng/mL)	74.58 ± 14.85
T_{max} (h)	0.5

Table 3. Stability of rivaroxaban (RRN) under various storage conditions (n = 6).

Storage condition	Nominal RRN concentration (ng/mL), A	Calculated RRN concentration (ng/mL), B	Bias of the mean of B compared with A (%)
Room temperature and four hours (sample extracts)	1.5	1.4 ± 0.2	-6.7
	400	451.4 ± 4.0	12.9
4°C and 12 hours (sample extracts)	1.5	1.5 ± 0.2	1.3
	400	420.3 ± 8.8	5.1

precision, and inter-day precision of 93.1-107.0%, 89.3-103.4%, <10.7%, and <12.7%, respectively (Table 1). QC sample were also used for determining recovery information of this method, and all mean recovery values calculated were within the range between 89.2 and 105.7% (Table 2). Finally, the bench-top stability and the autosampler stability of QC samples (LQC and HQC) were tested, and their limited bias values (the range of -6.7 to 12.9%) showed that RRN was stable enough for RRN analyses using the present method (Table 3). Since all validation parameters observed in this study were within acceptable criteria of the FDA guidelines, the present method is proven to be suitable for the determination of RNN in rat plasma.[14]

Application to pharmacokinetic studies in rats

The mean plasma concentration–time profile of RRN in rat plasma following oral administration of a RRN raw material in rats (a dose of 0.5 mg/kg) was obtained using the validated LC-MRM assay (supplementary information). As shown in Table 4, the validated method was suitable for determining the corresponding pharmacokinetic parameters of RRN such as $AUC_{0\text{-}24h}$, $AUC_{0\text{-}\infty}$, C_{max}, and T_{max}, and it is the additional evidence to show that the validated LC-MRM assay is good enough to quantitate RRN in rat plasma following the oral administration of a RRN formulation in rats.

Conclusions

A simple and efficient method to determine RRN in rat plasma using LLE and LC-MRM was developed. The use of ethyl acetate as the LLE solvent results appropriate extraction and purification of RRN and it also helps the significant reduction of rat plasma volume required for RRN quantitation. The developed method was successfully validated in various parameters including specificity, linearity, sensitivity, precision, accuracy, recovery, and stability by following the FDA guidelines. Finally, the applicability of the validated method to the PK evaluation of RRN was confirmed after its oral administration to normal rats. The present method is the first analytical

method employing LLE for the simple and efficient extraction and purification of RRN in rat plasma. Therefore, the present method can contribute to the development of new RRN formulations as well as to the monitoring of RRN in special clinical situations through its efficient determination in various samples with or without minor modification.

Supporting Information

Supplementary information is available at https://drive.google.com/file/d/1wIVHqqsb-sk6lh45_LWFW-hECuSFr70T/view?usp=sharing

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