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A Simple UPLC-MS/MS Assay of Rifampin in a Small Volume of Human Plasma

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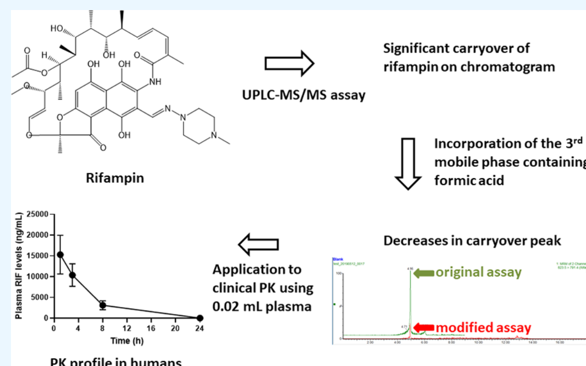
ABSTRACT: Rifampin (RIF) is a typical cytochrome P450 (CYP) 3A inducer and inhibitor of organic anion transporting polypeptide (OATP) 1B1 to assess drug−drug interaction (DDI) via CYP3A or OATP1B1 in clinical settings. To ensure sufficient exposure of RIF in DDI studies, it is important to determine plasma RIF concentrations. In this study, we developed a simple RIF assay in a small volume of human plasma by ultraperformance liquid chromatography with tandem mass spectrometry. RIF in 0.02 mL of plasma was extracted using protein precipitation and separated on a reverse phase column under gradient elution of three mobile phases, where the mobile phase C containing 1% formic acid was exclusively used to reduce the carryover of RIF. RIF and the internal standard were detected by multiple reaction monitoring in positive-ion electrospray ionization. RIF was quantifiable at 0.025−10 μg/mL without the carryover issue. The intra- and inter-run assays confirmed the reproducibility of the assay. Stability assessments ensured that RIF in human plasma was stable for 6 h at room temperature and for 409 days at −15 °C or below. The assay was successfully applied to a pharmacokinetic study with successful incurred sample reanalysis.



1. INTRODUCTION

Rifampin (RIF) has been used as a first-line therapeutic drug for tuberculosis. In order to make RIF efficacious, it is important to monitor the systemic exposure of RIF to higher than the minimum inhibitory concentration. It is also known that RIF induces cytochrome P450 (CYP) enzymes, and exposure itself may be lower by multiple doses. RIF has been used as a CYP3A inducer and recently also used as an inhibitor of organic anion transporting polypeptide 1B1 in clinical settings.[1] In the drug−drug interaction (DDI) studies mediated by CYP3A induction, the potential impact on exposure of investigational drugs was assessed by concomitant administration of RIF in clinical settings. It is thus important to monitor plasma RIF levels to ensure its exposure as a perpetrator in DDI studies. Various assays of RIF in human plasma have been reported so far including high-performance liquid chromatography (HPLC) with ultraviolet detection,[2,3] and liquid chromatography with tandem mass spectrometry (LC-MS/MS).[4−7] The maximum RIF concentrations in plasma are on the order of 10 μg/mL after an oral dose of RIF at 600 mg to humans[8] while the MIC for some Gram-positive bacteria was 1 ng/mL.[2] Thus, it is useful to develop an assay with a wider range to reduce the number of diluted samples for plasma RIF quantification. In addition, as RIF can be dosed to pediatrics along with adults, it is required to develop an assay with a smaller volume of plasma, given limitations in blood sampling. In the present study, we have developed a simple assay for the determination of RIF concentrations in human plasma by ultraperformance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS). The established assay has a wide quantification range of 0.025−10 μg/mL with a low plasma volume requirement of 0.02 mL. Potential risks of carryover were mitigated during assay optimization to maximize sensitivity, and the reproducibility of the established RIF assay was confirmed in the validation study using quality control (QC) samples and incurred sample reanalysis (ISR) using postdose samples.

2. METHODS

2.1. Reagents and Materials. RIF and the internal standard (IS), RIF-d8, were purchased from Merck KGaA (Darmstadt, Germany) and Alsachim SAS (Illkirch-Graffenstaden, France), respectively. Acetonitrile, distilled water, dimethyl sulfoxide (DMSO), and formic acid were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Ammonium formate was purchased from Thermo Fisher Scientific Inc. (Waltham, MA).

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2.2. Chromatographic and Mass Spectrometric Conditions. An ACQUITY UPLC H-Class Plus Bio system by Waters (Milford, MA) combined with a Xevo TQ-XS (Waters) as a quadrupole mass spectrometer was used as the UPLC-MS/MS system. RIF and the IS were separated on a reverse phase column, ACQUITY UPLC BEH C18 [2.1 mm inner diameter (i.d.) × 100 mm, 1.7 μm], with a gradient elution. The mobile phases consisted of (A) water-100 mmol/L ammonium formate (pH 5.0) (9:1, v/v), (B) acetonitrile-100 mmol/L ammonium formate (pH 5.0) (9:1, v/v), and (C) acetonitrile−water-formic acid (90:10:1, v/v/v). The flow rate and gradient condition of the mobile phases are represented in Table 1. To minimize the carryover of RIF in the assay, the mobile phase C was flushed exclusively from 5.01 to 9.0 min at a flow rate of 0.5 mL/min. The total run time was 13.5 min per assay. RIF and the IS were detected by multiple reaction monitoring in positive-ion electrospray ionization. The precursor/product ion mass transitions of RIF and the IS were m/z 823.5/791.4 and m/z 831.5/799.5, respectively. The mass spectrometry conditions were as follows: capillary voltage, 500 V; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 150 L/h; desolvation gas flow, 1000 L/h.

Table 1. Flow Rate and Gradient Condition for the Rifampin Assay

time (min)	mobile phase A (%)	mobile phase B (%)	mobile phase C (%)	flow rate (mL/min)
0	95	5	0	0.25
4.0	1	99	0	0.25
5.0	1	99	0	0.25
5.01	0	0	100	0.25
5.50	0	0	100	0.50
9.0	0	0	100	0.50
9.01	95	5	0	0.50
10.5	95	5	0	0.35
13.4	95	5	0	0.35
13.41	95	5	0	0.25
13.5	95	5	0	0.25

2.3. Preparation of Samples and Extraction Procedure. RIF was weighed and dissolved in DMSO to prepare a stock solution at 1 mg/mL. The stock solution was serially diluted to make working solutions of RIF. To prepare plasma calibration samples, the working solutions were spiked into blank human plasma (with K_2EDTA as an anticoagulant) at concentrations of 25, 75, 250, 500, 2500, 5000, 8000, and 10,000 ng/mL. Concentrations of RIF in QC samples were set at 25, 75, 500, and 8000 ng/mL for the lower limit of quantification (LLOQ), low concentration QC (LQC), mid concentration QC (MQC), and high concentration QC (HQC). The IS stock solution was prepared by dissolving RIF-d8 in DMSO at the concentration of 100 μg/mL, and then diluted with DMSO to make a working IS solution at 2000 ng/mL. The stock and working solutions of RIF and the IS were stored at 2−8 °C and were used within 409 days after preparation.

RIF in human plasma was extracted by using protein precipitation. To a 20 μL aliquot of plasma samples, 10 μL of the IS working solution (2000 ng/mL) was spiked and then 1.5 mL of acetonitrile was added to precipitate proteins. The resulting samples were vortexed and centrifuged at 15,700 × g for 5 min at 4 °C. A 0.5 μL aliquot of the supernatant was injected into the LC-MS/MS system for analysis.

2.4. Method Validation. A method validation study was performed in accordance with the bioanalytical guidelines from the European Medicines Agency[9] and the United States Food and Drug Administration.[10]

2.4.1. Linearity, Selectivity, Carryover. Linearity was evaluated by plotting the peak area of each calibration sample to the corresponding RIF concentrations. The calibration curve was subjected to linear regression with a weighting of 1/concentration^2. The relative error (RE) at each concentration of calibration samples was calculated, and the RE should be within ±15% (±20% was acceptable at the LLOQ) for 75% or more of the tested samples.

Selectivity of the assay was tested by using blank human plasma from six individuals. Blank plasma samples were extracted with the LLOQ samples to check whether any interfering peaks were detected at the retention times of the RIF and the IS on chromatograms. Areas of any interference peaks should be ≤20 and ≤5% of those of RIF and the IS, respectively, in the LLOQ sample.

Carryover was assessed by injecting a blank sample after the upper limit of quantification (ULOQ) across three batches. The same acceptance criterion used for the selectivity test was also applied to the carryover test.

2.4.2. Accuracy and Precision. Accuracy and precision were evaluated using QC samples at the following concentration levels: LLOQ, LQC, MQC, and HQC. Five replicates per concentration were tested and mean RE and RSD were calculated in the intrabatch reproducibility test. The intrabatch reproducibility tests were repeated three times as the interbatch reproducibility test across three separate days. The RE and RSD should be within ±15 and 15%, respectively, while ±20 and 20% were acceptable for LLOQ. To ensure dilution integrity of the assay, QC samples over the ULOQ at 25,000 ng/mL were diluted 10-fold with blank human plasma, and the RE and RSD of the diluted samples (five replicates) were determined.

2.4.3. Extraction Recovery and Matrix Effects. Extraction recovery in human plasma was determined at three concentrations, including low, mid, and high concentration levels, using the following equation.

$$\text{Extraction recovery (\%)} = \text{peak area of analyte in extracted plasma spiked before extraction} / \text{peak area of analyte in extracted blank plasma spiked after extraction} \times 100$$

The extraction recovery of the IS from human plasma was also evaluated at 1000 ng/mL. The acceptance criterion was not set, but the recovery should be consistent among the tested concentrations.

Potential matrix effects of RIF in human plasma were assessed at low and high concentrations by using plasma from six individuals. Matrix factors (MF) of RIF and the IS in plasma from six individuals were determined based on the equation below. The IS-corrected MF was then calculated by dividing the MF of RIF by the MF of the IS in each individual, and RSD of the IS-corrected MF should be within 15% to ensure minimal matrix effects.

$$\text{MF(\%)} = \text{peak area of RIF(or the IS) in extracted blank plasma spiked after extraction} / \text{peak area of RIF(or the IS) in neat solution} \times 100$$

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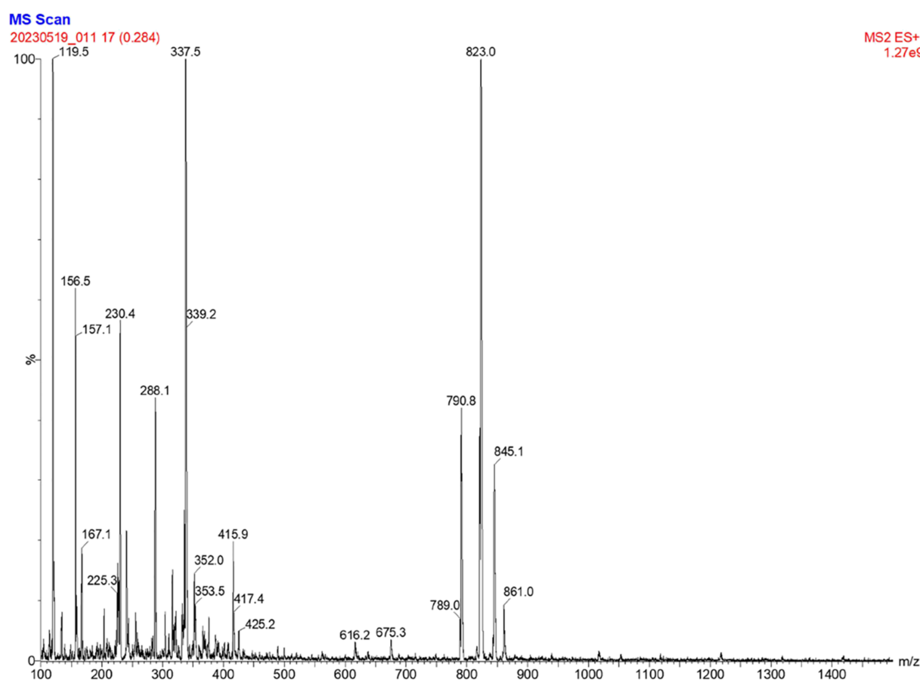

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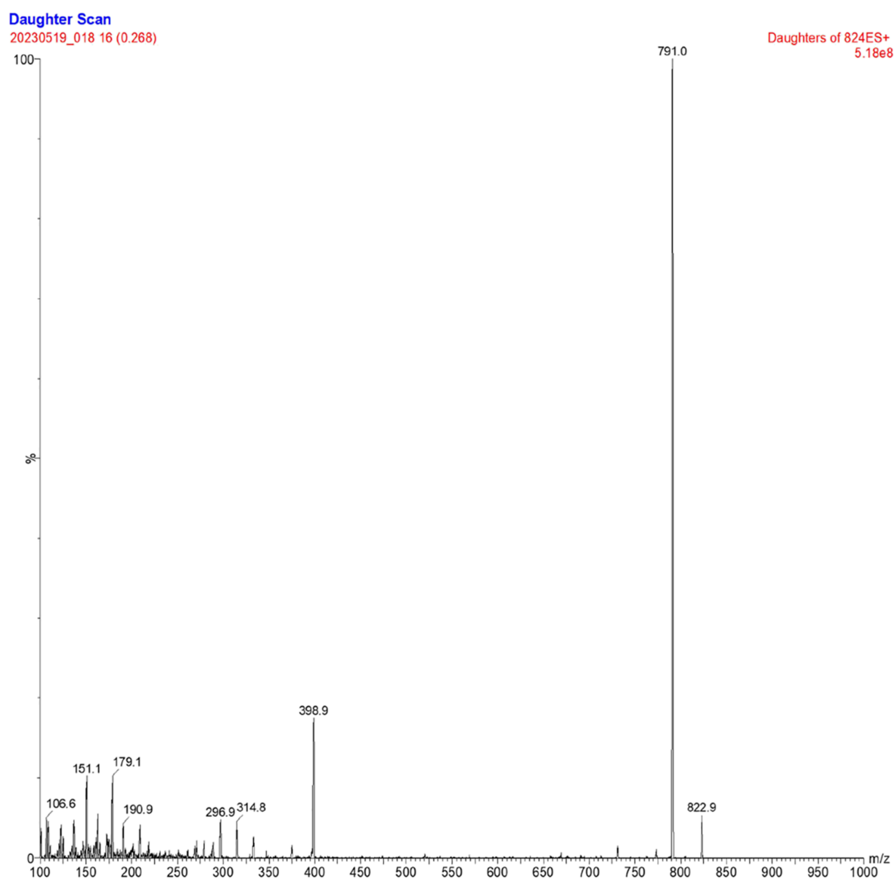

Figure 1. Representative mass spectrum of rifampin. (A) parent ions at m/z 100−1500. (B) product ions at m/z 823.5 with a collision energy of 25 V.

$$\text{IS} - \text{corrected MF (\%)} = \text{MF of RIF/MF of the IS} \times 100$$

2.4.4. Effects of Hemolysis and Hyperlipemia. Potential effects of hemolysis and hyperlipemia were evaluated using hemolyzed and hyperlipidemic plasma samples. Three replicates

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of QC samples per concentration were tested at low (75 ng/mL) and high (8000 ng/mL) concentrations. The RIF concentrations in the QC samples were calculated against calibration samples prepared from naive (nonhemolyzed or nonhyperlipidemic) plasma to calculate the RE. The mean RE of these samples should be within ±15% to ensure minimal impacts by hemolysis or hyperlipemia.

2.4.5. Stability. Benchtop, freeze−thaw, processed sample, and frozen stability in human plasma were assessed in triplicate using LQC and HQC concentrations. Bench-top stability in plasma was assessed at ambient temperature for 6 and 24 h. Freeze−thaw stability in plasma was tested after five cycles. The stability of the RIF in processed samples was assessed at 4 °C for 6 days. Frozen stability in plasma was tested at −15 °C or below for 409 days. The concentrations in stored samples were determined, and the mean RE of the stored samples should be within ±15% to ensure stability. Bench-top stability was also assessed in human whole blood at low (50 ng/mL) and high (5000 ng/mL) concentrations. RIF was spiked in blank human whole blood and stored on a bench for 2 h at room temperature. The stored blood samples were centrifuged to obtain plasma samples. The stability of the RIF in standard solutions was also tested. The working and stock solutions at 50 ng/mL and 1 mg/mL, respectively, were stored refrigerated (2−8 °C) for 409 days or on the bench for 24 h. The peak intensity of RIF in both human whole blood and working stock solutions was determined, and the residual percentage of the stored solution was calculated by comparing the stored samples with that of the sample at time = 0. The residual percentage should range from 85 to 115%.

2.5. Application to a Clinical Study. The established assay of RIF was applied to a clinical PK study. Fourteen subjects were enrolled in the study with written consent. RIF was orally administered at 600 mg once daily for 7 days. On day 7, RIF at 600 mg was orally administered with E7090 (35 mg oral dose), and blood samples were serially obtained from each subject at 1, 3, 8, and 24 h postdose. Blood samples were centrifuged at 3000 rpm for 10 min at 5 °C and plasma samples as a supernatant were collected in tubes. Plasma samples in tubes were stored frozen at −15 °C or below until analysis. The plasma samples were assayed within the stability duration ensured in this study. Three plasma samples were reassayed in the ISR assessment to ensure reproducibility of the assay. Plasma RIF concentrations in the reassay should be within ±20% of those in the original results.

3. RESULTS

3.1. Method Development. During method optimization, the most abundant precursor ion of RIF was m/z 823 in positive ion mode. The precursor ion m/z 823 yielded various product ions and the most intense one was m/z 791, which was used as the optimized transition of RIF (Figure 1).

The stability of RIF in stock solution was first assessed by dissolving the standard in methanol, which was kept on the bench for 24 h at ambient temperature. An interference peak at m/z 821, which was considered to be a rifampin quinone, increased in intensity over time, making it the most predominant precursor ion. By changing the solvent of the standard solution to DMSO from methanol, the intensity of the unknown peak decreased and was comparable to that at time = 0. These findings led us to select DMSO as the solvent for the standard stock solution. Similar observations occurred when methanol-acetonitrile (1:1, v/v) was first used as the extraction solvent; therefore, acetonitrile was selected.

We first checked the linearity of the calibration curve up to 20 μg/mL given the maximum RIF levels in plasma at a 600 mg oral dose. The peak intensity of RIF at 20 μg/mL in calibration samples did not increase linearly with the concentration when compared to the peak intensity at 10 μg/mL. Although the RE at 20 μg/mL was still within ±15%, it was more negatively biased compared to that at the other lower concentrations. The ULOQ of the calibration curve was thus set at 10 μg/mL.

3.2. Method Validation. *3.2.1. Linearity, Selectivity, and Carryover.* In the linearity test, RIF was quantifiable from 25 to 10,000 ng/mL with RE within ±15%. The correlation of determination of the calibration curve in multiple assay batches was >0.99. These findings indicated that linearity of RIF in this assay was ensured. The selectivity of the assay was confirmed in blank plasma samples from six individuals. RIF and the IS were eluted on chromatograms at the retention times of approximately 4.4−4.6 min (Figure 2).

No interference peaks were detected at the retention times of RIF and IS. Areas of carryover peaks at the retention time of RIF were less than 20% of those of the LLOQ in three separate assay batches, indicating minimal carryover.

3.2.2. Accuracy and Precision. RE as accuracy and RSD as precision were estimated in the intra- and interassay batch reproducibility tests. At the four QC concentrations (LLOQ, LQC, MQC, and HQC), RE and RSD were within ±15 and 15%, respectively (Table 2). The RE and RSD of diluted QC samples by 10-fold were −5.3 and 0.6%, respectively, which met the acceptance criteria. This finding indicated that it is possible to accurately assay RIF even by a 10-fold dilution.

3.2.3. Extraction Recovery and Matrix Effects. Extraction recoveries of the RIF and the IS from human plasma were determined. The extraction recoveries of RIF were 86.6, 86.2, and 87.8% at low, mid, and high concentrations, respectively. The recoveries were sufficiently high and comparable among the three concentrations tested (Table 3). The recovery of IS was determined at 1000 ng/mL in the presence of RIF at three concentrations. The recoveries of the IS ranged 98.6−99.2%. The extraction recovery of the IS was sufficiently high and similar to that of RIF.

The MFs of RIF and the IS in human plasma under the present assay conditions were determined at low and high concentrations using blank human plasma from six individuals. The MF of RIF was 98.1 and 100.2%, at low and high concentrations, respectively (Table 2). The MF of RIF was consistent at the two concentrations tested. The MF of IS was 98.7 and 99.8% at low and high concentrations of RIF, respectively. The MF of the IS was similar to that of RIF. The mean IS-corrected MF of RIF was 99.4 ± 1.3 and 100.5 ± 0.3% at low and high concentrations, respectively, with the RSD of 1.3 and 0.3%, respectively. These findings indicated minimal matrix effects.

3.2.4. Effects of Hemolysis and Hyperlipemia. Potential effects of hemolysis or hyperlipemia in plasma on the RIF assay were evaluated at low and high concentrations. The RE of QC samples prepared from hemolyzed plasma was 1.1 and 0.8% at low and high concentrations, respectively. The RE of QC samples prepared from hyperlipidemic plasma was −1.3 and 0.8% at low and high concentrations, respectively. These findings indicated that RIF was accurately assayed even in hemolyzed or hyperlipidemic plasma.

3.2.5. Stability. The stability in human plasma was assessed at low and high concentrations, and the results are presented in Table 4. Results in the benchtop stability in human plasma were

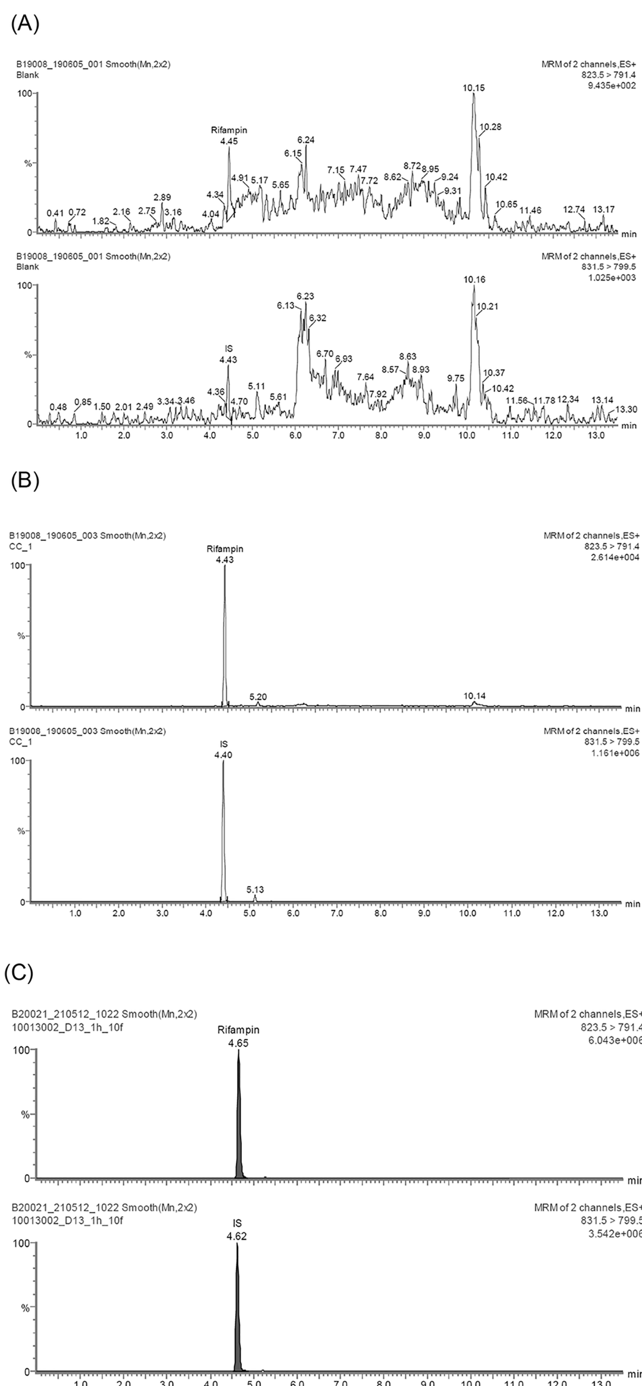

Figure 2. Representative chromatograms of rifampin in human plasma. (A) Blank plasma, (B) LLOQ, and (C) postdose sample collected 1 h after the oral dose of rifampin at 600 mg. LLOQ: lower limit of quantification.

ensured up to 6 h, but not for 24 h. The stability in human plasma after five freeze−thaw cycles was confirmed with temperature changes from −15 °C or below to room temperature. Processed sample stability was ensured for 6 days at 4 °C. Frozen stability in human plasma was ensured up to 409 days when stored at −15 °C or below. The stability in human whole blood was confirmed for 2 h at room temperature. RIF was stable in the stock and working solution for 409 days when stored at −15 °C or below and for 24 h when stored on a bench at ambient temperature.

Table 2. Intra- and Inter-Run Accuracy and Precision for the Determination of Rifampin in Human Plasma[a,b]

QC	concentration (ng/mL)	RE (%)	RSD (%)
intrarun			
LLOQ	25	−4.5	4.2
LQC	75	−8.9	1.8
MQC	500	−5.7	1.1
HQC	8000	−11.5	3.6
inter-run			
LLOQ	25	−4.6	3.0
LQC	75	−8.1	5.4
MQC	500	−6.4	5.8
HQC	8000	−8.6	7.9

[a]HQC: high concentration quality control (QC); LLOQ: lower limit of quantification; LQC: low concentration QC; MQC: mid concentration QC; QC: quality control; RE: relative error; RSD: relative standard deviation. [b]Data represents the mean RE and RSD of 5 replicates and 15 replicates in the intra- and inter-run reproducibility, respectively.

3.3. Application to a Clinical Study. RIF was orally administered to 14 subjects at 600 mg for 7 days once a day, and blood samples were collected on day 7. Plasma concentrations were determined using the validated assay. Plasma RIF concentrations reached the maximum at 1 h postdose and declined thereafter (Figure 3).

The ISR was conducted by using six plasma samples. Plasma RIF concentrations in the ISR samples were within ±20% of those in the original assay (−2.6 to 10.9%). This finding indicates that the ISR met the acceptance criteria and further supported that the assay was reproducible.

4. DISCUSSION

The assay of RIF in human plasma was developed by using UPLC-MS/MS with only 20 μL of plasma with a quantification range of 25−10,000 ng/mL. Carryover was often reported when developing assays with a wider quantification range, and indeed in the method development, carryover of RIF was evident when blank samples were injected just after the injection of the ULOQ sample. The peak area of the interfering peaks was 158% of that of the LLOQ sample, which was out of the acceptance criterion. A preliminary study indicated that the inclusion of formic acid at low levels in the mobile phase reduced the carryover. However, addition of formic acid in the mobile phases A and B lowered the peak intensity of RIF. Therefore, the third mobile phase C which comprised the organic solvent [acetonitrile−water = 9:1 (v/v)] with 1% formic acid was flushed from 5.01 to 9.0 min, at which RIF elution was completed. In the modified elution using the three mobile phases, the peak area of interfering peaks was reduced to 16% of that of the LLOQ sample with minimal effects on the intensity of RIF (Figure S1).

Our study focuses on widening the quantification range, minimizing the plasma volume used for the assay and simplifying the extraction procedure. These three points are important for pharmacists who are involved in the therapeutic drug monitoring of rifampin. Wider quantification ranges reduce the chances to dilute plasma samples to fit the rifampin concentrations in plasma samples within the quantifiable range. The lower plasma volume required for the assay is very helpful for pediatrics and senior patients who are suffering from repeated collection of a large volume of blood samples. The LLOQ in this study (25 ng/mL) may seem higher given the

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Table 3. Mean Extraction Recoveries and Matrix Factors of Rifampin and the Internal Standard in Human Plasma[a,b]

analyte	concentration (ng/mL)	% mean recovery	MF	IS-corrected MF
rifampin				
LQC	75	86.6 ± 1.0	98.1 ± 1.0	99.4 ± 1.3 (1.3)
MQC	500	86.2 ± 3.3	N.T.	N.T.
HQC	8000	87.8 ± 1.6	100.2 ± 1.0	100.5 ± 0.3 (0.3)
IS	1000 (75)	98.6 ± 1.0	98.7 ± 0.6	N.A.
	1000 (500)	99.2 ± 1.6	N.T.	N.A.
	1000 (8000)	98.7 ± 0.9	99.8 ± 0.8	N.A.

[a]HQC: high concentration quality control (QC); IS: internal standard; LQC: low concentration QC; MF: matrix factor; MQC: mid concentration QC; N.A.: not available; N.T.: not tested. [b]Data in extraction recoveries represent the mean ± standard deviation of three replicates at each concentration. Data in the MF represent the mean ± standard deviation of human plasma from six individuals. The figures in parentheses of the IS concentration represent the rifampin concentrations present in the mixture. The figures in parentheses of the IS-corrected MF represent the relative standard deviation of six individuals.

Table 4. Stability of Rifampin in Human Plasma[a,b]

stability test	condition	QC	RE (%)
bench-top in plasma	room temperature for 6 h	LQC	−11.9
		HQC	−7.6
bench-top in whole blood	room temperature for 2 h	LQC	2.4
		HQC	4.3
freeze/thaw in plasma	5 cycles	LQC	−7.7
		HQC	−9.4
long-term in plasma	−15 °C or below for 409 days	LQC	−10.7
		HQC	−7.7
processed samples	4 °C for 6 days	LQC	−1.9
		HQC	−5.6

[a]HQC: high concentration quality control (QC); LQC: low concentration QC; QC: quality control; RE: relative error. [b]Data represents mean RE from the nominal concentration in LQC and HQC samples stored under the designated conditions (n = 3 each). Rifampin concentrations in LQC and HQC samples were 75 and 8000 ng/mL, respectively, except for benchtop stability in whole blood (50 and 5000 ng/mL, respectively).

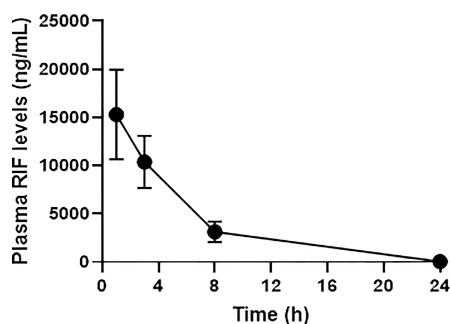

Figure 3. Plasma concentration−time profile of rifampin in humans. Data represent the mean ± standard deviation of 14 subjects. Rifampin was repeatedly orally administered at 600 mg for 7 days, and blood samples were serially collected on day 7 from each subject up to 24 h.

lower LLOQ of other therapeutics quantified by the LC-MS/MS; however, 25 ng/mL as the LLOQ is sufficient for sensitivity to monitor rifampin levels at therapeutic doses in the drug−drug interaction study, and pursuing the lower LLOQ is not the scope of this study. As it is important to set the LLOQ given therapeutic dose and efficacious drug levels, the LLOQ in this study is considered sufficient given efficacious rifampin levels in human plasma. Indeed, in this study, the plasma levels of rifampin were measured up to 24 h postdose, which enables estimation of pharmacokinetic parameters of rifampin if necessary. Although this study was performed to support a clinical drug−drug interaction study of E7090 with rifampin as a CYP3A4 perpetrator, the assay itself can also be applicable to nonclinical studies. It is very important in nonclinical settings to reduce plasma volume for the assay since a limited volume of blood samples can be obtained from animals (especially in rodents) than humans. In this sense as well, the developed assay in this study can also be useful in nonclinical settings in which a large volume of blood sampling is limited. The established assay used a very simple protein precipitation by an organic solvent. Previous publications used other extraction procedures with higher complexity such as solid phase extraction.[11] The established method achieved sufficient sensitivity with a simple extraction procedure, which is more advantageous than the other precedent methods. Regarding the selectivity, it is generally believed that UPLC-MS/MS methods had minimal issues in selectivity once the analyte was retained in an analytical column. Indeed, the theoretical plate and retention factor of rifampin in the established assay were approximately 90,000 and 4, respectively, which indicates sufficient separation characteristics. Thus, it is likely that comedications are eluted separately from rifampin on an analytical column, even when the comedications are present in plasma samples and simultaneously assayed. In addition, it is likely that minimal matrix effects are expected with the use of a stable isotope labeled IS even when other drugs are eluted at the same retention times as rifampin. The molecular weights of the frequently used first-line antituberculosis drugs are approximately 123, 137, 204, and 581 for pyrazinamide, isoniazid, ethambutol, and streptomycin, respectively, which are different from that of rifampin. The reported mass transitions of the drugs were different from that of rifampin[4] and thus minimal interferences by these drugs are expected in this assay even if the drugs elute at the same retention time of rifampin. Given these considerations, it is highly likely that other complications impact the determination of rifampin concentrations in the established assay.

Although the established assay is useful in the assay of rifampin in terms of a wider quantification range and simple extraction with a lower volume of plasma samples, there are some limitations. The run time of the present assay is 13.5 min, which is longer than the previous assays.[6,11−13] The run times of rifampin in the reported assays were 2.4 min,[12] 3.6 min,[6] 7 min,[11] and 9 min,[13] which can achieve higher throughput in the assay than the present assay. In addition, this assay uses a relatively complex gradient program of the mobile phases under the chromatographic conditions to address the carryover issue for achieving the wider quantification range.

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In the stability assessment using human plasma, benchtop stability was evaluated for 6 and 24 h at ambient temperature. The RE was −32.8 and −18.0% at low and high concentrations, respectively, when stored for 24 h, which was out of the acceptance criterion. When stored for 6 h, the RE was within ±15%, which indicated that RIF is stable in human plasma for at least 6 h but not for 24 h when stored at room temperature. This is consistent with the previous finding that RIF was not stable at 0.125 μg/mL up to 12 h in human plasma even with vitamin C on a bench.[3] However, conflicting results were published, where benchtop stability was ensured for 1 day[6] or 12 h[4]) in human naive plasma. It was also reported that RIF was stable in human naive plasma up to 2 h at 1 μg/mL on the bench but not for 4 h.[2] Other studies assessed shorter duration in the benchtop stability in human naive plasma (2 h,[7] 4 h[5]) but it is not evident that longer stability than the reported one was assessed. Reasons for the discrepancies in the stability results in human plasma remain to be clarified; however, potential differences in characteristics of human naive plasma used in the laboratories (e.g., the storage period of plasma under frozen conditions) and/or in actual temperature of room temperature when stability was assessed may account for the discrepancies. In addition, it may be partially attributed to the fact that the stability of RIF in human plasma on a bench was concentration-dependent. Le Guellec et al. reported that RIF in human plasma was stable up to 8 h at 10 μg/mL while only up to 2 h at 1 μg/mL when stored on a bench.[2] The benchtop stability results in this study also suggested that the stability of RIF in human plasma was concentration-dependent; the RE at LQC was lower than that at HQC (−32.8 vs −18.0%) when stored for 24 h. Although conflicting results have been reported in the benchtop stability of RIF in human plasma, it is better to keep the duration of plasma samples on the bench (e.g., extraction) as short as possible to accurately determine RIF levels in human plasma.

5. CONCLUSIONS

A simple method for the RIF assay in human plasma was developed by using UPLC-MS/MS with only 0.02 mL of human plasma samples. The carryover issue of RIF in the original assay was addressed by the optimization of the mobile phase. The assay complied with the bioanalytical guidelines, and the results of validation parameters met the predefined acceptance criteria that was recommended. The assay was applied to a clinical PK study of RIF and the ISR results further supported the reproducibility of the assay.

■ ASSOCIATED CONTENT

Data Availability Statement

Data will be made available on reasonable request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c04814.

Additional experimental details and results, including chromatographic conditions and chromatograms (PDF)

Reduction of carryover peak of rifampin by modification of the HPLC conditions with chromatograms and the original and modified assay conditions (PDF)

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Author Contributions

T.S.: Conceived and designed the experiments; performed the experiment; analyzed and interpreted the data; reviewed the manuscript. T.I.: conceived and designed the experiments; analyzed and interpreted the data; reviewed the manuscript. K.H.: conceived and designed the experiments; analyzed and interpreted the data; reviewed the manuscript. Y.M.: conceived and designed the experiments; analyzed and interpreted the data; wrote the manuscript.

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Notes

The authors declare no competing financial interest.

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The study protocol of this study was approved by the Institutional Review Board of Kitasato University Shirokane Campus (Tokyo, Japan). A written consent was obtained from all the subjects before starting the study. The study was confirmed that the clinical trial was complied with all the relevant regulations.

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