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Validated Liquid Chromatography-Electrospray Ionization Tandem Mass Spectrometric Method for Quantification of Rifaximin Human Plasma for Pharmacokinetic Study: A Randomized, Open-Label, Two-Period, Comparative Crossover Study in Healthy Korean Male Volunteers

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Abstract: *A sensitive and specific liquid chromatography combined with electrospray ionization (ESI) tandem mass spectrometry (LC-MS/MS) method, operating in the positive ionization mode, for quantifying of rifaximin in human plasma using rifaximin-d_6 as internal standard (IS) was developed and validated. The analyte and IS were extracted by simple one step liquid to liquid extraction (LLE). The chromatographic separation was performed on a Gemini C_{18} column (50 X 2.0 mm, 5 um) under isocratic conditions using a mixture of acetonitrile / 10 mM ammonium formate (in 0.1% formic acid) (80:20, v/v) as mobile phase at a flow rate of 0.20 ml/min. Quantitation was performed using multiple reaction monitoring (MRM) mode to study parent → product ion transitions of m/z 786.4 → 754.3 for rifaximin and m/z 792.5 → 760.4 for IS, respectively. Linearity in plasma was obtained over the concentration range 10 ~ 5000 pg/ml, with a coefficient of determination (r^2) of 1.000. The method was has been successfully applied for routine assay to support pharmacokinetic study of rifaximin in human plasma after an oral administration of 200 mg rifaximin.*

Keywords: *Rifaximin, Human plasma, LC–MS/MS, Pharmacokinetics*

I. Introduction

The chemical name for rifaximin is (2S,16Z,18E,20S,21S,22R,23R,24R,25S,26S,27S,28E)-5,6,21,23,25-pentahydroxy-27-methoxy-2,4,11,16,20,22,24,26-octametyl-2,7-(epoxypentadeca[1,11,13]trienimino)benzofuro(4,5-e)pyrido[1,2-a]-benzimidazole-1,15(2H)-dione,25-acetate, empirical formula is $C_{43}H_{51}N_3O_{11}$ and its molecular weight is 785.9 (Fig. 1). It is a semisynthetic, rifamycin-based non-systemic antibiotic owing to its poor oral bioavailability. Rifaximin is used to treat traveler's diarrhea that is caused by a bacteria called Escherichia coli. It is also used to prevent hepatic encephalopathy, which is a condition that occurs when our liver does not work normally [1, 2].

Several methods including HPLC-UV, HPLC coupled with electrochemical detection (ECD) and radioligand binding technique have been reported for the determination of rifaximin. However, lack of sensitivity hindered the application of HPLC-UV on the pharmacokinetic study of rifaximin [3-7]. Furthermore, laborious and time-consuming procedures of sample preparation and long analysis time also restricted its application.

Therefore, a highly sensitive, advanced high throughput LC-MS/MS method was developed and validated for the determination of rifaximin in human plasma. This developed method offered higher sensitivity, simpler sample treatment procedure, smaller sample volume requirement and shorter run time of 2.5 min.

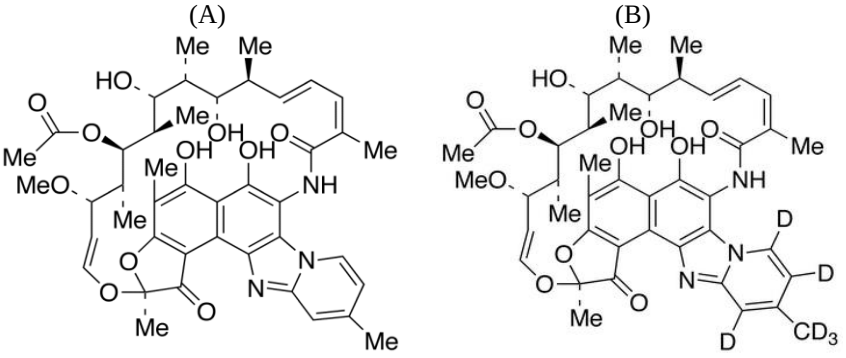

Fig. 1. Structural representation of rifaximin (A) and deuterated rifaximin-d_6 (B).

II. Experimental

2.1. Materials and Instrument

Rifaximin (99.0%) and rifaximin-d_6 hydrochloride (IS, 96.0%) were purchased from Sigma-Aldrich Co., LLC. (USA) and Santa Cruz Biotechnology, Inc. (USA), respectively. Acetonitrile (J.T. Baker, USA) was HPLC grade, and other chemicals were of analytical grade. All aqueous solutions including the buffer for the mobile phase were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. Drug-free plasma for the preparation of calibration standard was obtained from metro-hospital blood donor service (Anyang, Korea). Before analysis, the blank samples were analyzed by the present LC-MS/MS method. No significant peaks were observed at the retention times of the analyte and IS.

An Agilent 1200 system consisting of G1312A quaternary pump, G1379B degasser, G1367B auto-sampler, G1330B thermostat,G1316A column oven (TTC) compartment (Agilent, Waldbronn, Germany) was used for solvent and sample delivery. An API 5000 triple-quadrupole mass spectrometer equipped with a Turbo Ion Spray (ESI) source was used for mass analysis and detection (Applied Biosystems, Foster City, CA, USA). Data processing was performed on Analyst 1.6 software package.

2.2. Chromatographic and Mass Spectrometric Conditions

Isocratic chromatographic separation was achieved on a Gemini C_{18} column, 50 mm X 2.0 mm, i.d., 5 um (Phenomenex, USA). The mobile phase consisted of acetonitrile / 10 mM ammonium formate (in 0.1% formic acid) (80:20, v/v) at a flow rate of 0.20 ml/min. The column and auto-sampler temperature were maintained at 35 ℃ and 10 ℃, respectively.

The mass spectrometer was operated in positive ionization mode. The tuning parameters were optimized for rifaximin and the IS by infusing a solution containing 500 ng/ml of both analytes at a flow rate of 10 ul/min into the mobile phase (0.20 ml/min) using analyte column 'T' connection. Optimized instrument settings specific rifaximin and IS were as follows: curtain gas was 20 psi, ion source gas 1 was 50 psi, ion source gas 2 was 50 psi, ionspray voltage was 5500 V, turbo heater temperature was 600 ℃. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions m/z 786.4 → 754.3 for rifaximin and m/z 792.5 → 760.4 for the IS, respectively, with a dwell time of 150 ms per transition. The precursor ions of rifaximin and IS were formed using declustering potentials of 141 and 161 V, respectively, and their precursor ions were fragmented at collision energies of 31 and 31 eV by collision-activated dissociation with nitrogen at a pressure setting of 5 (arbitrary units). Both quadrupoles were maintained at unit resolution.

2.3. Preparation of Calibration Standard and QC Samples

A stock solution of rifaximin with a concentration of 1.0 mg/ml (calculated from purity) was prepared by dissolving 10.1 mg of rifaximin in 10 ml of methanol. Six standard working solutions of 100, 500, 1000, 5000, 10000 and 50000 pg/ml of rifaximin were made by further dilution of the stock solution with 50% methanol. The quality control (QC) samples were similarly prepared at concentrations of 300, 20000 and 40000 pg/ml, by a separate weighing of the pure standard. A 25000 pg/ml working solution of the IS was also prepared by diluting the 0.1 mg/ml stock solution of rifaximin-d_6 with 50% methanol.

Matrix-matched calibration standards and QC samples of rifaximin were prepared by spiking 30 ul of the working solutions into 270 ul of drug-free plasma. The calibration standards were prepared at concentrations of 10, 50, 100, 500, 1000 and 5000 pg/ml of rifaximin in plasma, while the corresponding QC samples were prepared at 30, 2000 and 4000 pg/ml.

These standard-spiked plasma calibration solutions and QC samples were stored at −20 ℃. For each batch of unknown samples to be analyzed, the appropriate standard and QC solutions were brought to room temperature, and processed through the plasma sample preparation procedure in parallel with the unknown samples.

2.4. Sample preparation

To 10 ml polypropylene tube, 30 ul of the IS solution (rifaximin-d_6, 25000 pg/ml) was added to 300 ul of plasma sample and vortexed for 10 sec. The plasma sample was then maintained to be basic condition by addition of 100 ul of 0.1 N phosphoric acid. Rifaximin and IS were extracted with 3 ml of methyl tert-butyl ether for 10 min on vortex-mixer. The organic and aqueous layers were separated by centrifugation at 4000 rpm for 5 min. The organic layer was transferred to another clean tube and evaporated under nitrogen gas at 50 ℃ and the dry contents reconstituted with 100 ul of 80% acetonitrile and vortex mixed for 30 sec. The contents were finally transferred into appropriate auto-sampler vials and an aliquot (5 ul) was injected onto the LC-MS/MS for analysis.

2.5. Method validation

Plasma samples were quantified using the ratio of the peak area of analyte to IS as the assay response. The specificity of the method was determined by analyzing six different batches of human plasma as is, to

demonstrate the lack of chromatographic interference from endogenous plasma components. Sets of spiked calibration curve (CC) standards and QC samples (n=4 at each concentration) were prepared and analyzed on three different occasions to evaluate linearity, precision and accuracy. To evaluate linearity, plasma calibration curves were prepared and assayed on three consecutive days over the range of 10 ~ 5000 pg/ml. Least-squares linear regression was used for curve fitting with $1/x^2$ as the weighting factor. For determining the intra-day precision and accuracy, a replicate analysis of plasma samples of rifaximin in human plasma was performed on the same day. The run consisted of a CC and five replicates of each the lower limit of quantification (LLOQ), low, mid and high concentration QC samples. The inter-day precision and accuracy were assessed by analysis of three batches on different days. The precision was expressed as the coefficient of variation (CV %) and the accuracy as the relative error (R.E. %). The extraction recovery of the analytes from the plasma was evaluated by comparing the mean detector responses of three replicates of processed QC samples at low and high concentration to the detector responses of standard solutions of same concentration. Endogenous matrix components may change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector. Three set of samples were prepared by directly spiking the analytes into reconstitution solution and without the presence of residue extracted from blank plasma. Post-preparative stability, three aliquots each of low and high QC samples were stored at 10 ℃ in an auto-sampler for 24 hr, analyzed and the concentrations were compared with the actual values. Three aliquots of each low and high QC samples were kept in deep freezer at -70 ℃ for 36 day. The samples were processed and analyzed and the concentrations obtained were compared with the actual value of QC samples to determine the long term stability of analyte in human plasma. Three aliquots each of low and high unprocessed QC samples were kept at ambient temperature (25 ℃) for 19 hr in order to establish the short term stability of the analytes. The stability of the analytes after three freeze and thaw cycles was determined at low and high QC samples. The samples were stored at -70 ℃ for 24 hr and thawed unassisted at room temperature. After completely thawing, the samples were refrozen for 12 ~ 24 hr. After three freeze-thaw cycles, the concentration of the samples were analyzed. Separate standard working solutions containing 300 pg/ml, 40000 pg/ml of rifaximin and 25000 pg/ml of IS were prepared and stored at 25 ℃ and 2 ~ 8 ℃ for 20 hr and 17 day. The response obtained from the two drugs was calculated and compared with that of the freshly prepared solutions of the same concentration.

2.6. Pharmacokinetic study

The validated method was used to determine the plasma concentrations of rifaximin from a clinical trial in which 52 healthy male volunteers received a single oral dosage of 200 mg rifaximin. Eligible volunteers were Korea men aged 19 to 48 years (26.8 ± 5.2) and the average body weight was 69.1 ± 9.2 kg. The study protocol was approved by the Human Investigation Ethics Committee of Metro hospital, Anyang-ci, Korea. Blood samples were collected into heparinized glass tubes before and 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 3, 4, 6, 8, 12 and 24 hr post-dosing, and centrifuged at 4000 rpm (4 ℃) for 10 min to separate the plasma fractions. The collected plasma samples were stored at −70 ℃ until analysis.

Determination of the pharmacokinetic parameters was performed by non-compartmental assessment of data using the computer program K-BE Test 2007 (ver. 1.1.0, KFDA, Korea). Mean and individual concentration–time profiles were generated and used to determine the maximum plasma concentration (C_{max}) and the time to attain these maximum concentrations (T_{max}). The area under the plasma concentration–time curve from time zero to the time of the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The terminal elimination rate constant (ke) was estimated by log-linear regression of concentrations observed during the terminal phase of elimination.

III. Results and Discussion

3.1. Optimization of the mass spectrometric condition

Mass parameters were tuned in both positive and negative ionization modes for the analytes. Good response was achieved in positive ionization mode. By positive electrospray ionization (ESI) mode, the detector was operated at unit resolution in the multiple-reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of rifaximin at *m*/*z* 786.4 → 754.3 and IS at *m*/*z* 792.5 → 760.4. Optimized parameters were as follows: curtain gas, gas 1 and gas 2 (nitrogen) 20, 50 and 50 units, respectively; dwell time 150 ms; source temperature 600 ℃; ion spray voltage 5500 V. Declustering potential and collision energy were 141 V and 31 eV for rifaximin and 161 V and 31 eV for IS, respectively.

3.2. Optimization of the chromatographic condition

In pursuit of symmetric peak shape and retention time of ~0.92 min, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH range of 3.0 ~ 7.0, along with altered flow rates (in the range of 0.2 ~ 0.5 ml/min) were tested for complete chromatographic resolution of rifaximin and IS (data not shown). The resolution of peaks

was achieved with 10 mM ammonium formate (in 0.1% formic acid) and acetonitrile mixture (20:80, v/v) with a flow rate of 0.2 ml/min, on a Gemini C_{18} column and was found to be suitable for the determination of electrospray response for rifaximin and IS.

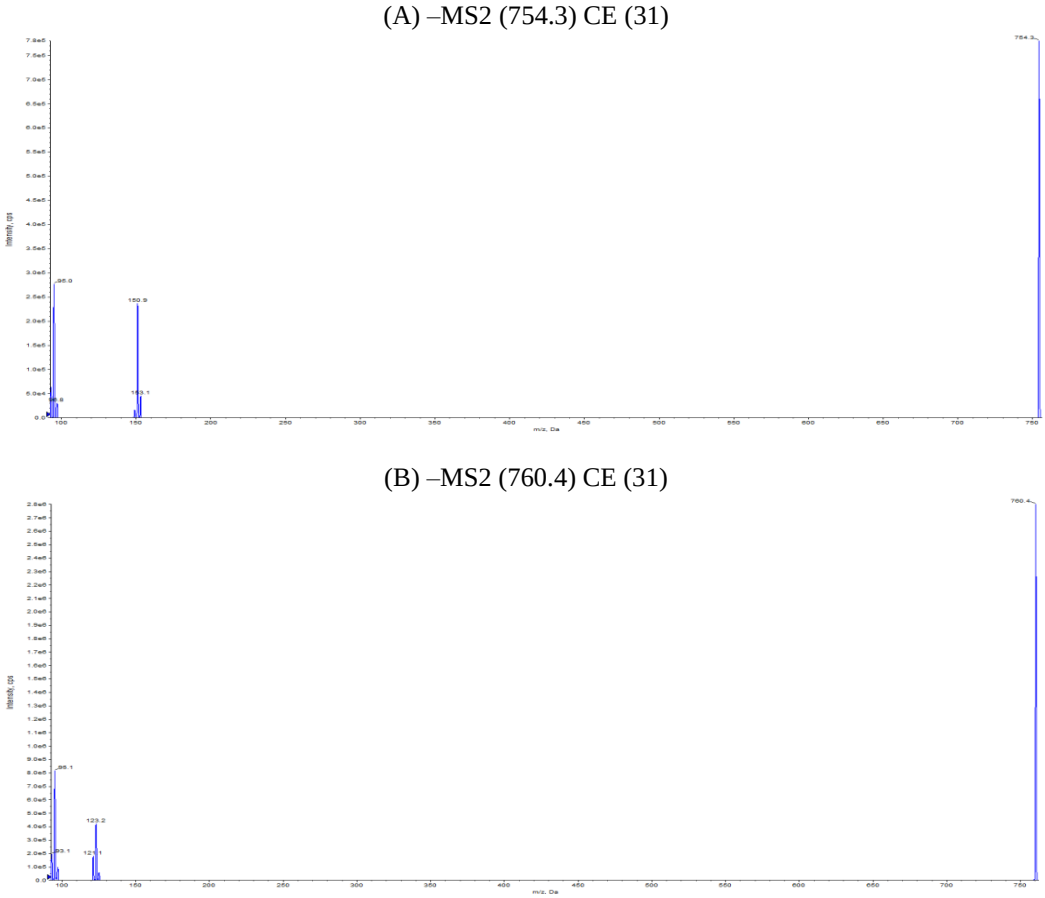

Fig. 2. Product ion spectra of $[M\text{-}M]^+$ of rifaximin (A) and rifaximin-d_6 (B).

3.3. Sample pre-treatment

In order to achieve cleanliness in extract, liquid to liquid extract (LLE) was optimized for extraction of analytes from plasma. The extraction solvent (methyl tert-butyl ether) gave consistent results in terms of recovery of rifaximin and IS and also gave cleaner plasma blank samples.

3.4. Assay specificity

A typical chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with rifaximin at LLOQ are shown in Fig. 3, respectively. No interfering peaks from endogenous compounds are observed at the retention times of analytes and IS. The retention time of rifaximin and IS was 0.92 and 0.91 min. The total chromatographic run time was 2.5 min.

3.5. Linearity and Lower limit of Quantification

The linear regression of the peak-area ratios versus concentrations was fitted over the concentration range of 10 ~ 5000 pg/ml in human plasma. A typical equation of the calibration curves was as follows: y = 0.0004x - 0.0037 (r^2=1.000), where y represents the peak-area ratio of analyte to IS and x represents the plasma concentration of rifaximin. Good linearity was seen in this concentration range.

The lower limit of quantification was 10 pg/ml for determination of rifaximin in plasma. The precision and accuracy at the concentration of LLOQ are shown in Table 1.

3.6. Precision and Accuracy

The method showed good precision and accuracy. Table 1 summarizes the intra- and inter-day precision and accuracy for rifaximin from QC samples. The intra-day precision (CV %) for QC samples (10, 30, 2000, 4000 pg/ml) were 2.49%, 2.23%, 0.54% and 0.97%, respectively and that of inter-day analysis were 4.64%, 4.67%, 1.44%, 1.85% with a relative errors (R.E. %) within 0.40% to 6.20%.

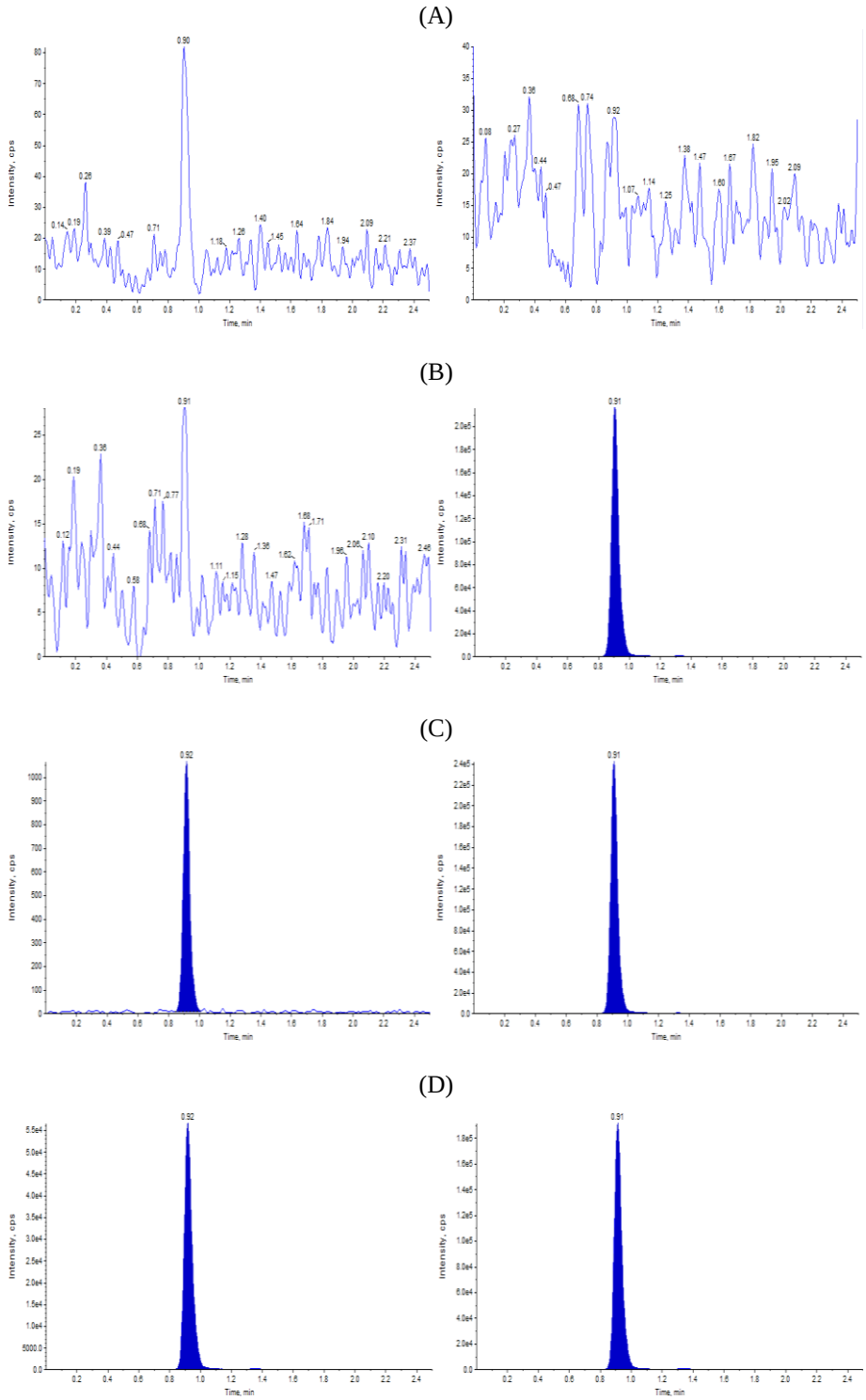

Fig. 3. Typical MRM chromatograms of rifaximin (left panel) and IS (right panel) in (A) human blank plasma, (B) human blank plasma spiked with IS, (C) human plasma spiked with rifaximin at LLOQ (10 pg/ml) and IS, (D) a representative chromatogram (1 hr) of extracted a male volunteer.

3.7. Recovery and Matrix Effect

The extraction recoveries of rifaximin from human plasma were 93.71% (CV=4.57%), 90.64% (CV=2.75%) and 96.01% (CV=1.01%) at concentration levels of 30, 2000 and 4000 pg/ml, respectively, and the mean extraction recovery of IS was 91.11% (CV=1.76%).

The endogenous components are mainly the cause of ion suppression effects during electrospray ionization. The extent of this effect is mainly dependent on sample extraction procedure and is also compound dependent. The result indicated that the matrix components did not alter or deteriorate the performance of the

proposed method as the % coefficient of variation (CV %) of two QC samples was less than 97.21% and 97.85% for rifaximin and IS respectively indicates the reproducibility of peak area as well as the extracts were 'clean' and no unseen component interfere with the ionization of the analytes. The extraction recoveries and matrix effect on the estimation of the analytes was shown in Table 2.

3.8. Stability

The result of stability experiments showed that no significant degradation occurred during the chromatography, extraction and sample storage of rifaximin plasma samples. Stability data are shown in Table 3.

Table 1. Precision and accuracy data for the analysis of rifaximin in human plasma (n=5).

Added (pg/ml)	Intra-day			Inter-day		
	Found (pg/ml)	CV (%)	R.E. (%)	Found (pg/ml)	CV (%)	R.E. (%)
10	10.62	2.49	6.20	10.07	4.64	0.65
30	30.21	2.23	0.69	28.96	4.67	3.46
2000	2007.95	0.54	0.40	1975.25	1.44	1.24
4000	4133.54	0.97	3.34	4048.29	1.85	1.21

Table 2. Extraction recovery and matrix effect of rifaximin and rifaximin-d_6 (IS).

Analyte	Concentration (pg/ml)	Recovery (%)	Matrix effect (%)
Rifaximin	30	93.71 (CV=4.57%)	97.21 (CV=1.86%)
	2000	90.64 (CV=2.75%)	-
	4000	96.01 (CV=1.01%)	98.90 (CV=1.10%)
Rifaximin-d_6	2500	91.11 (CV=1.76%)	97.85 (CV=1.99%)

Table 3. Summary of stability of rifaximin in human plasma under various storage conditions (n=3).

Storage conditions	Concentration (pg/ml)			CV (%)	Variation (%)
	Added	Initial	After		
Post preparative (24 hr at 10 ℃)	30	28.93	27.30	2.78	-5.65
	4000	4090.52	3908.60	1.05	-4.45
Short-term (19 hr at 25 ℃)	30	28.93	26.91	0.91	-6.98
	4000	4090.52	3924.82	0.66	-4.05
Long-term (36 day at -70 ℃)	30	28.93	28.78	1.22	-0.54
	4000	4090.52	4002.11	0.75	-2.16
Three freeze/thaw (3 cycles)	30	28.93	29.07	5.67	0.48
	4000	4090.52	4142.23	0.56	1.26
Stock solution (20 hr at 25 ℃)*	30	7913	8107	3.55	2.45
	4000	1094176	1074705	8.76	-1.78
Stock solution (17 day at 2 ~ 8 ℃)*	30	7913	7789	4.49	-1.57
	4000	1094176	1088422	1.50	-0.53

* Stability of stock solution was evaluated peak area.

3.9. Application in Pharmacokinetic Study

This validated analytical method was applied to investigate the pharmacokinetic profiles of rifaximin in human plasma after an oral administration of 200 mg rifaximin. Profile of the mean plasma concentration of rifaximin versus time is shown in Fig. 4. The main pharmacokinetic parameters of rifaximin in 50 volunteers were calculated.

For the pharmacokinetic analysis of plasma, the mean (SD) values obtained for the test and reference products were as follows: C_{max}, 991.56 (351.15) and 1012.57 (350.31) pg/ml; T_{max}, 1.09 (0.37) and 1.19 (0.48) hr; $T_{1/2}$, 7.96 (2.10) and 8.50 (1.92) hr; AUC_{0-t}, 5040.46 (1002.58) and 5260.72 (1194.20) pg·hr/ml; and $AUC_{0-\infty}$, 5633.84 (1079.20) and 5974.63 (1419.45) pg·hr/ml, respectively (Table 4). The parametric 90% confidence intervals for AUC_{0-t}, and C_{max} values were 91.76 ~ 101.64% and 89.79 ~ 106.74%, respectively, and were entirely within the bioequivalence acceptance limits.

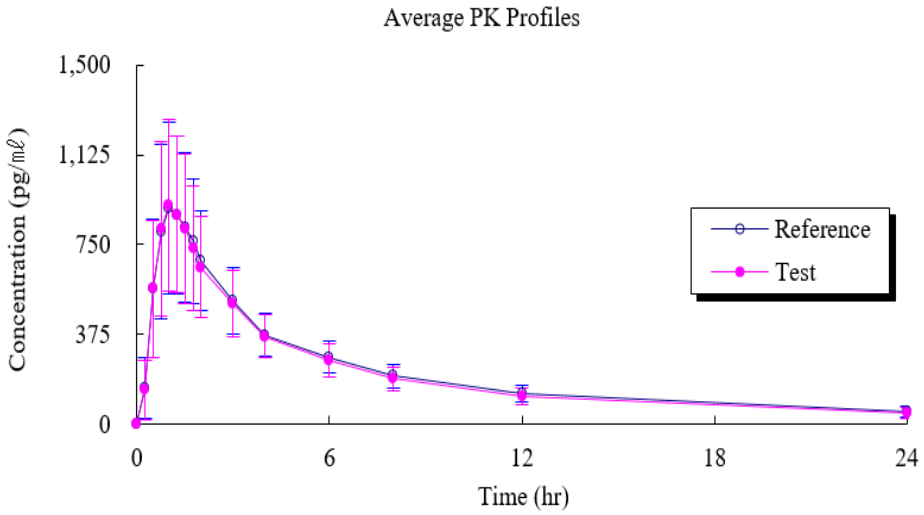

Fig. 4. Mean plasma concentration–time curve of rifaximin after a single oral dose of 200 mg rifaximin to 50 healthy male volunteers. Each point represents the mean ± SD.

Table 4. Pharmacokinetic parameters of rifaximin after a single oral dose 200 mg rifaximin tablets of the test and reference.

Parameters	Test*	Reference**
C_{max}, pg/ml	991.56 ± 351.15	1012.57 ± 350.31
T_{max}, hr	1.09 ± 0.37	1.19 ± 0.48
AUC_{0-t}, pg·hr/ml	5040.46 ± 1002.58	5260.72 ± 1194.20
$AUC_{0-\infty}$, pg·hr/ml	5633.84 ± 1079.20	5974.63 ± 1419.45
$t_{1/2}$, hr	7.96 ± 2.10	8.50 ± 1.92

* Nexpharm rifaximin Tab., 200 mg (Nexpharm Korea Co., Ltd. Korea)
** Normix Tab., 200 mg (Sam-oh Pharm Co., Ltd. Korea)

IV. Discussion

To the best of our knowledge, we have developed for the first time fully validated LC–MS/MS method for the determination of rifaximin, which provides the highest sensitivity (10 pg/ml) using a simple LLE procedure. A good internal standard should track the analyte during extraction and any inconsistent response due to matrix effect. This is also established with almost the same recovery of IS compared to the analyte. The most appropriate IS for typical anions are none other than deuterated compounds and hence rifaximin-d_6 was used as IS. Results obtained by usage of d_6 internal standard were consistent and reproducible which was evident by incurred sample analysis conducted on this study.

V. Conclusions

An LC-MS/MS assay for rifaximin in human plasma was developed and validated with respect to linearity, precision and accuracy, and analysis of real samples was demonstrated. It was proved to be superior in sensitivity, sample pretreatment and speed of analysis in comparison to the previously reported analytical methods. This method was successfully applied to pharmacokinetic studies for rifaximin and was found to be sensitive and reliable.

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