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Sensitive quantification of rifaximin in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatography–mass spectrometry method (LC–MS/MS) for the quantitative determination of rifaximin in human plasma was developed and validated. In the developed procedure, metoprolol was added to human plasma as an internal standard (IS) and acetonitrile was used to precipitate the plasma proteins before LC–MS/MS analysis. Chromatographic separation was obtained on a RESTEK Pinnacle C18 column (50 mm × 2.1 mm, 5 μm) with a mobile phase consisted of ammonium acetate solution (15 mM, pH 4.32) as buffer A and methanol as mobile phase B. Quantification was performed in positive mode using multiple reaction monitoring (MRM) of the transitions *m/z* 786.1 → 754.1 for rifaximin and *m/z* 268.3 → 116.1 for the IS. The assay has been validated over the concentration range of 0.5–10 ng/ml ($r = 0.9992$) based on the analysis of 0.2 ml of plasma. The assay accuracy was between 98.2% and 109%. The within-day and between-day precision was better than 3.9% and 8.9% at three concentration levels. The freeze–thaw stability was also investigated and it was found that both rifaximin and the IS were quite stable. This method provides a rapid, sensitive, specific and robust tool for the quantitative determination of rifaximin in human plasma, which is especially useful for the pharmacokinetic study of rifaximin.

Keywords: Rifaximin; Metoprolol; LC–MS/MS; Human plasma

1. Introduction

Hepatic encephalopathy (HE) is a complex hepatic disease. Its pathological cause remains speculative, but increasingly it is regarded as a metabolic or neurophysiologic disorder [1]. Rifaximin (Fig. 1) is one of the non-absorbable antibiotics for the treatment of HE because it is not absorbed from the gastroenteric mucosa and persists longer at higher levels in the lumen of the gastroenteric tract, minimizing the risk of systemic side effects [2]. The antimicrobial spectrum of rifaximin includes aerobic and anaerobic Gram-positive and Gram-negative bacteria [3,4]. It is used primarily to treat local conditions within the gastrointestinal tract. After oral administration it undergoes virtually no systemic absorption [5]. In a recent pilot study, rifaximin provided some clinical benefits for patients with steroid-refractory severe ulcerative colitis [6]. Due to its non-absorbable characteristic, pharmacokinetic studies in animals and also in human showed that its concentration in body fluid was rather low [7–9], especially in plasma, almost all the concentrations was under the limit of detection (LOD). However, sensitivities of previous reports were not high enough. Therefore, these conclusions need to be further examined with a more sensentive method. This requirement prompted us to develop a more sensitive quantification method to determine the plasma concentrations of rifaximin.

Several methods including HPLC–UV [9–11], HPLC coupled with electrochemical detection (ECD) [7,12] and radioligand binding technique [13] have been reported for the determination of rifaximin. However, lack of sensitivity hindered the application of HPLC/UV on the pharmacokinetic study of rifaximin. Furthermore, laborious and time-consuming procedures of sample preparation and long analysis time also restricted its application. HPLC–ECD was another choice for the quantification of rifaximin. Although its relatively high

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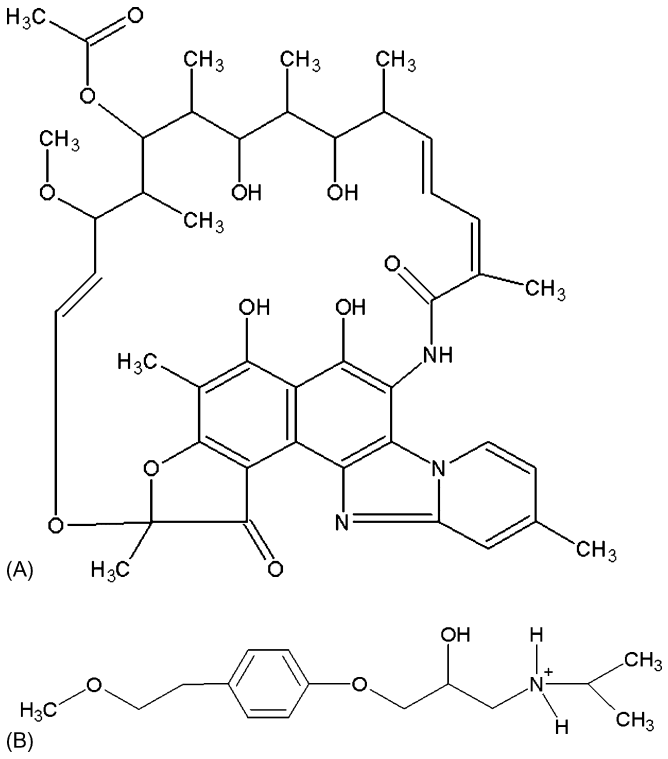

Fig. 1. Chemical structures of (A) rifaximin (B) metoprolol (the IS).

sensitivity allowed the achievement of a limit of quantification of 2 ng/ml [7], there were many practical issues, such as difficulties in stabilizing the detector, interferences by endogenous peaks, and restriction of mobile phases, associated with this method. The technique of radioligand binding was recently reported [13], but the technique was difficult to perform and the procedures were rather laborious. Moreover, most laboratories prefer not to handle radiomaterials. On the other hand, modern pharmacokinetic studies requires methods that can accurately measure low concentration levels of drugs in small amounts of available samples in a high throughput fashion. Therefore, there is an unmet need to develop a rapid, sensitive, and robust analytical method for the quantitative analysis of rifaximin in plasma.

The objective of the present investigation was to develop an LC–MS/MS method to accurately and rapidly determine the plasma concentrations of rifaximin. Recently, LC–MS/MS was used widely to measure concentrations of various drugs in biological fluids and tissues because of its high specificity, sensitivity, and robustness. High selectivity of MS/MS detection has greatly simplified the process of sample preparation and method development. Therefore, the LC–MS/MS technique combined with rapid sample preparation by simple protein precipitation seemed to be the best choice for the quantification of plasma rifaximin. So far, to our knowledge, the quantitative determination of human plasma levels of rifaximin by reversed-phase LC–MS/MS has not been reported in the literature. In our method, metoprolol (Fig. 1B) was employed as the IS. The developed method provides a highly sensitive and selective way to determine the levels of rifaximin in human plasma with a lower limit of quantification of 0.5 ng/ml with only 0.2 ml of plasma. This method was validated and applied successfully to determine the plasma concentrations of rifaximin of clinical patients treated with this drug.

2. Experimental

2.1. Chemicals and reagents

Rifaximin was provided by Sanye Pharm. Com. (Hainan Province, China). Metoprolol was purchased from National Institute for the Control of Biological Products (Beijing, China). Methanol (Fisher Chemical, USA), ammonium acetate (Dikma Technologies Inc., USA) and acetonitrile (Dikma Technologies Inc., USA) of HPLC grade were used. The mobile phase was filtrated with a 0.45 μm film before use. Drug-free human plasma was purchased from the local blood bank (Beijing Red Cross Blood Center, China) and was stored at −40 °C. The water used was distilled and deionized.

2.2. Instrumentation

LC–MS/MS analysis was performed with an Agilent 1100 HPLC system (Agilent Technology, USA) consisting of binary pumps, an autosampler and a vacuum degasser. The HPLC system is coupled to an API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray (TIS, Applied Biosystems, Foster City, CA) source, under the control of the Analyst software (Version 1.4).

2.3. Rifaximin calibration standard and sample preparation

Stock solutions of rifaximin (1 mg/ml) and the IS (0.4 mg/ml) were prepared in methanol and stored at 4 °C. Seven rifaximin working solutions (5, 10, 20, 40, 60, 80 and 100 ng/ml) were made by diluting the stock solution with methanol. The IS working solution was 480 ng/ml. All the working solutions were prepared freshly before use.

For the preparation of plasma samples, 20 μl of the IS working solution was added to a 1.5 ml polypropylene tube containing 200 μl of plasma spiked with rifaximin and vortex-mixed for 10 s. Then 600 μl of acetonitrile was added, vortex-mixed for another 1.0 min, and centrifuged for 5 min at 12,000 rpm. The clear solution was transferred to a brown sample vial for injection.

2.4. Chromatography conditions

Chromatographic separation was made by reverse-phase HPLC on a RESTEK Pinnacle C18 column (50 mm × 2.1 mm, 5 μm). The mobile phase consisted of 15 mM ammonium acetate, pH 4.32 as buffer A and methanol as mobile phase B. The column was run at a flow rate of 0.2 ml/min from 50% B to 100% B over 3 min. The mobile phase was then returned to the original 50% B at 3 min and remained at 50% B for an additional 2 min. The injection volume was 5 μl.

2.5. Mass spectrometry conditions

The positive ion detection mode was used. Nitrogen was the nebulizer and curtain gas. Collision induced dissociation was achieved using nitrogen as the collision gas. The ion source conditions were set as following: temperature 400 °C, nebulizer gas 5 units (units refers to an arbitrary value set by the Analyst software), curtain gas 9 units, collision gas 5 units, ionspray voltage 5000 V, collision energies 33.0 V for rifaximin and 27.0 V for the IS, electron multiplier voltage 2100 V, declustering potential 54 V for rifaximin and 55 V for the IS, focusing potential 400 V, entrance potential 10 V, collision exit potential 27 V for rifaximin and 9 V for the IS, and the dwell time 200 ms. Under these conditions, transitions m/z 786.1 → 754.1 was used for the detection of rifaximin and m/z 268.3 → 116.1 for the IS.

2.6. Calibration and validation

The calibration plasma samples at levels of 0.5, 1, 2, 4, 6, 8 and 10 ng/ml were precipitated with acetonitrile and analyzed by the described LC–MS/MS method. Calibration graphs ($Y = aX + b$) were constructed by plotting the relative peak area rifaximin/IS (Y) versus analyte concentration (X) in ng/ml, with a weighting factor $1/X$.

The accuracy and intra-batch precision of the method were determined by analyzing the quality control samples (QC) for five times at three different concentrations, that is, 1, 4 and 8 ng/ml. To determine the between-batch precision, the QC samples of three different batches were analyzed and each level was analyzed for five times in parallel. The freeze–thaw stability was also examined by freezing the QC samples at −40 °C over night and thawing them at room temperature for three cycles.

2.7. Application to clinical samples

A test study was done to determine the plasma levels of rifaximin in clinical patients who were treated with 200 mg of rifaximin. Blood samples were collected following the proper guidelines set up by the hospital. Blood from 10 clinical patients was collected before drug administration and 1, 2 and 4 h after drug administration. This is a test study, not a full course pharmacokinetic study, what we care about is the maximum concentration, and therefore blood samples were collected only to 4 h after drug administration. Plasma samples were stored at −40 °C until analysis. After addition of 20 μl IS to 0.2 ml plasma samples, they were precipitated with acetonitrile and analyzed by LC–MS/MS.

3. Results and discussion

3.1. MS/MS detection

Initially, both the positive and negative modes of detection were investigated and we found that the positive mode of detection was more sensitive for rifaximin and for metoprolol. Hence, positive mode was employed in the experiments. In this mode, the very soft ionization process in the TIS source produces the precursor ions $[M+H]^+$. The major precursor ions observed were $m/z = 786.1$ for rifaximin and $m/z = 268.3$ for the IS. Each of the precursor ions was subjected to collision induced dissociation in order to obtain product ions. The collision conditions were optimized and it was found that the best detection was obtained when the collision gas was set at 5 units, the collision energy was at 33 V for rifaximin and 27 V for the IS. Under these conditions, the full product ion spectra of rifaximin and the IS were acquired (Fig. 2). The fragmentation mechanism was also proposed based on the product spectra and their structures. After collision, the dissociation of rifaximin results in one main fragment of m/z 754.1, suggesting a possible loss of methanol molecule (Fig. 3A). Therefore, ion transition of m/z 786.1 → 754.1 was selected for the detection of rifaximin. For the IS, the process of dissociation was much more complex. The collision produced more complicated fragments and several steps were likely involved in the process [14]. The probable fragmentation mechanism for the IS are proposed in Fig. 3B to account for most of the observed product ions. The ion transition m/z 268.3 → 116.1, which is the strongest product ion, was selected for the detection of the IS.

3.2. Optimization of the chromatography conditions

Several analytical columns of different sizes and brands were evaluated to found out which one produced the sharpest and most symmetrical peaks. We observed that a RESTEK Pinnacle C18 column (50 mm × 2.1 mm, 5 μm) provided satisfying peak

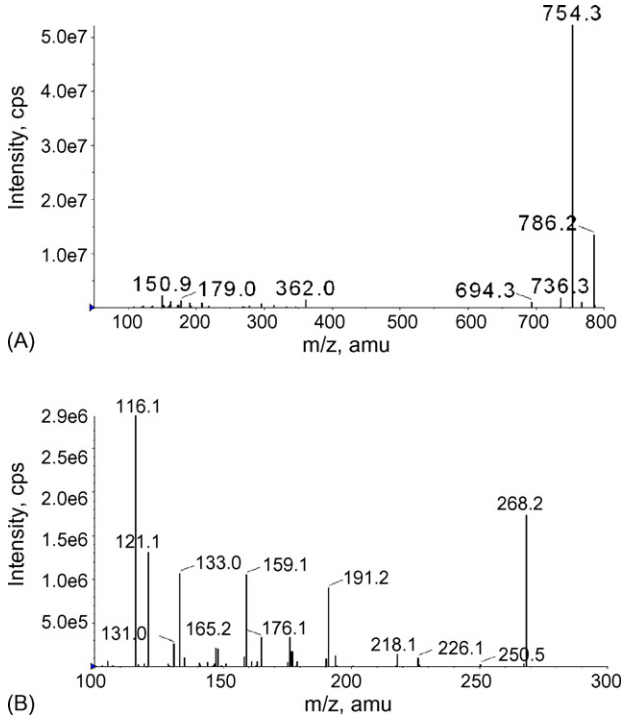

Fig. 2. Representative full-scan product ion spectra of rifaximin 50–800 amu (A) and of the IS 100–300 amu (B).

shapes and was hence employed in this study. The mobile phases were also optimized. A gradient mobile phase consisting of ammonium acetate buffer (15 mM, pH 4.32, A) and methanol (B) was found to produce adequate retention and good peak shapes for rifaximin and for the IS. The column was eluted at a flow rate of 0.2 ml/min from 50% B to 100% B over 3 min. Then it returned to 50% B and remained there for another 2 min. The chromatograms for rifaximin and the IS are depicted in Fig. 4. As shown, the retention times of rifaximin and the IS were 2.26 and 1.23 min, respectively. The total analysis time was 5 min for each run.

3.3. Method validation

3.3.1. Specificity

Drug-free plasma was precipitated by acetonitrile and analyzed by the developed method. Fig. 5 shows the representative chromatograms for a drug-free plasma sample. No endogenous peaks are present at the retention times (t_R) of rifaximin or that of the IS, demonstrating that the developed LC–MS/MS method is highly selective. Moreover, that no analyte-interfering peaks were observed in the 10 patient samples that were collected before drug administration also supports the high selectivity of this method. Peaks at the expected retention time did show up in the plasma samples of some patients who were administered with the drug (Fig. 6).

3.3.2. Linearity, precision and accuracy

The weighted regression calibration ($1/X$) curve was linear over the concentration range of 0.5–10 ng/ml in human plasma. The mean equation of linearity was $Y = (0.0832 \pm 0.0164)X + (0.0179 \pm 0.0113)$ ($n = 5$), with each of the correlation coefficients (r) greater than 0.9990. There was less than 12.0% deviation between the nominal and experimental concentrations calculated from the equations (Table 1). The limit of detection (LOD) was 0.16 ng/ml and the limit of quantification (LOQ) was 0.5 ng/ml.

An assessment of intra-batch and between-batch precisions were conducted by analyzing QC samples at three levels. Data of precisions are presented in Table 2. The intra-batch and the between-batch precisions ranged from 2.2 to 3.9% and 5.7 to 9.1%, respectively. Accuracies for intra-batch and between-

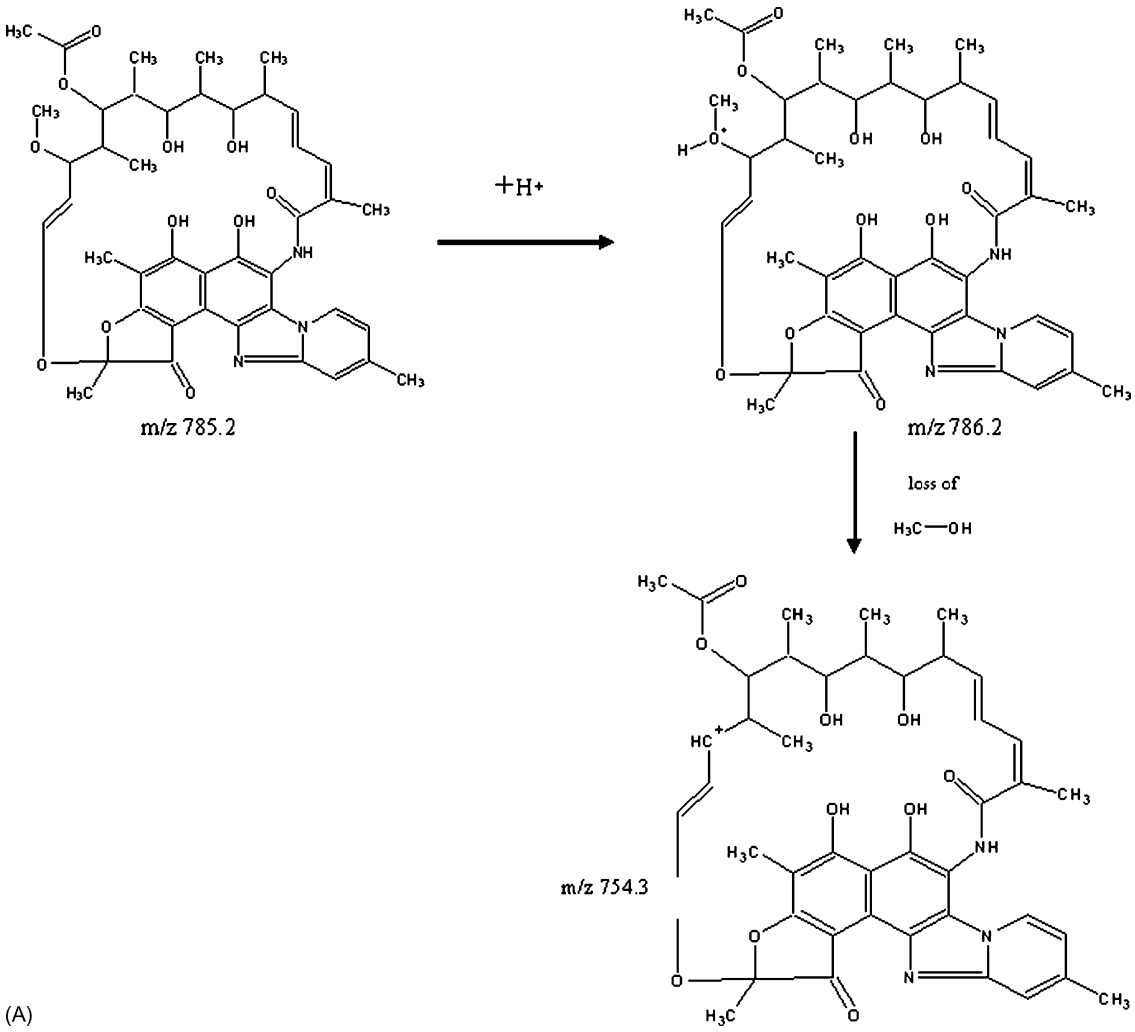

Fig. 3. Proposed fragmentation pathways of (A) rifaximin (B) the IS.

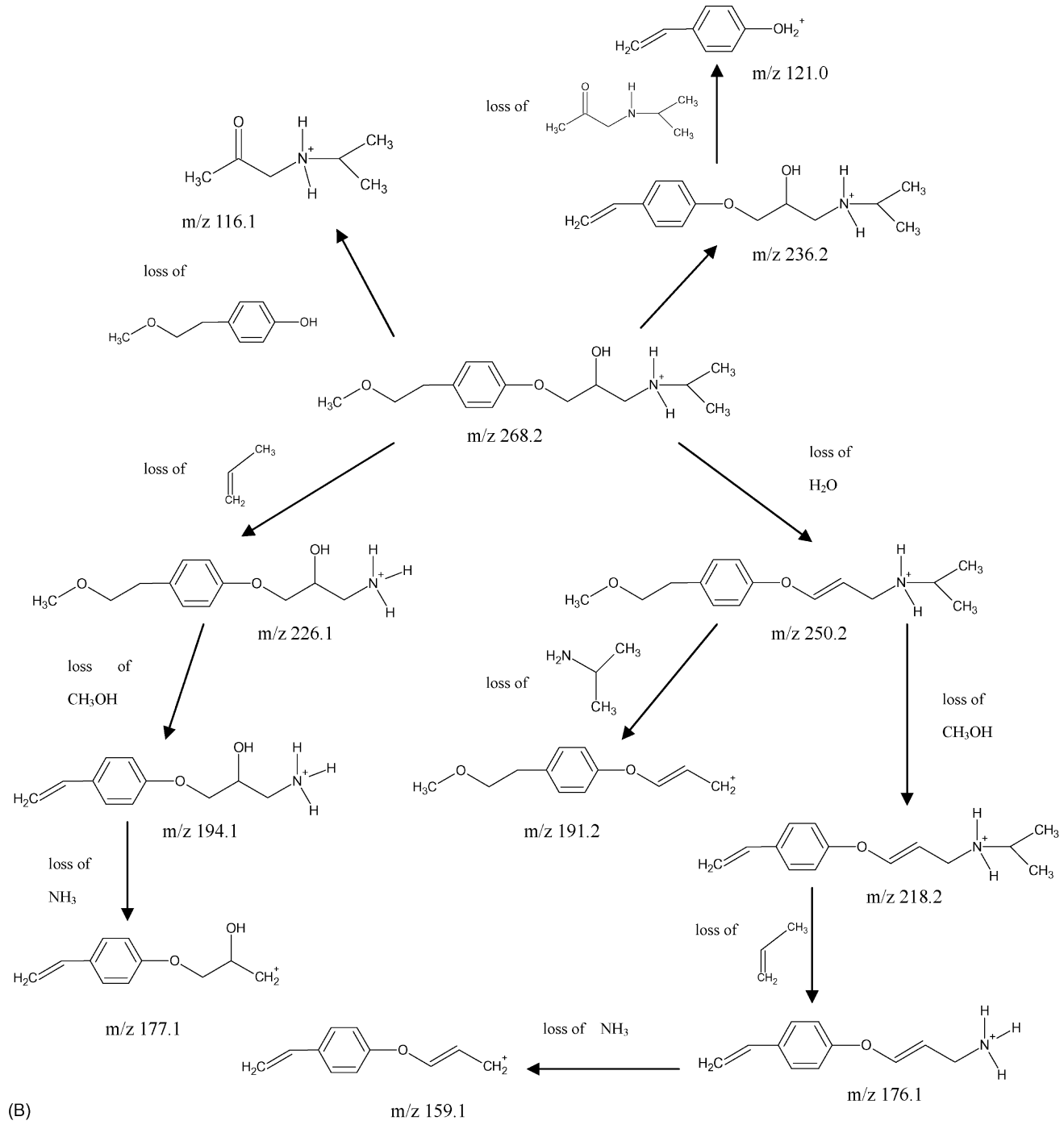

Fig. 3. (*Continued*).

batch samples were 101–109% and 98.2–108%, respectively, of the nominal value.

3.3.3. Robustness

There is a continuing demand for bioanalytical methods based on LC–MS/MS systems for the evaluation of new chemical entities because of its specificity, sensitivity, robustness, and high-throughput capability. Matrix interference on ionization and detection is likely a problem when protein precipitation is the only step used for sample preparation as compared to liquid–liquid extraction and solid-phase extraction methods. In spite of this concern, the protein precipitation method has been employed as the routine sample preparation procedure for LC–MS/MS assays in our laboratory mainly due to its simplicity and robustness. Matrix effect was not a problem for us. The sensitivity and accuracy of our method are satisfying for the purpose of our study (Table 2). However, further sample clean up by solid phase extraction along with sample concentration might increase the sensitivity even more.

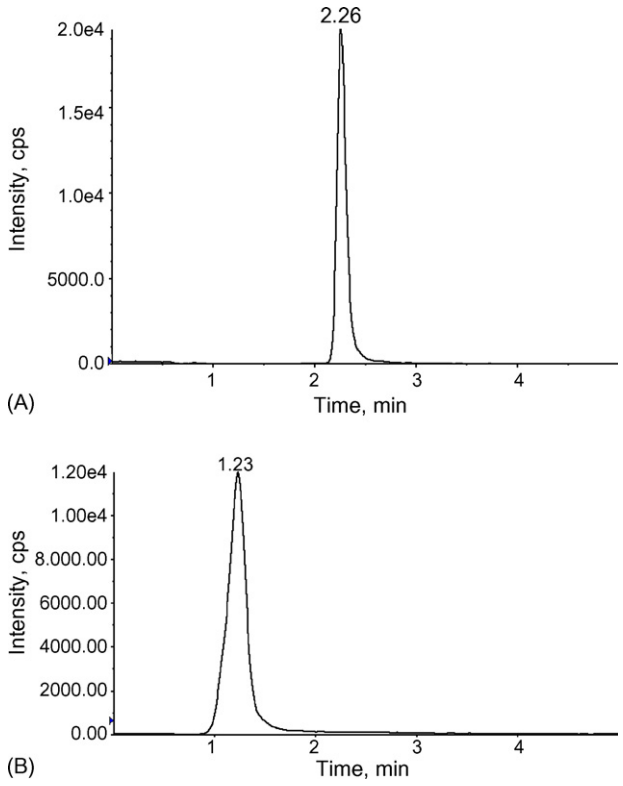

Fig. 4. Chromatograms of standard samples (A) rifaximin (B) the IS.

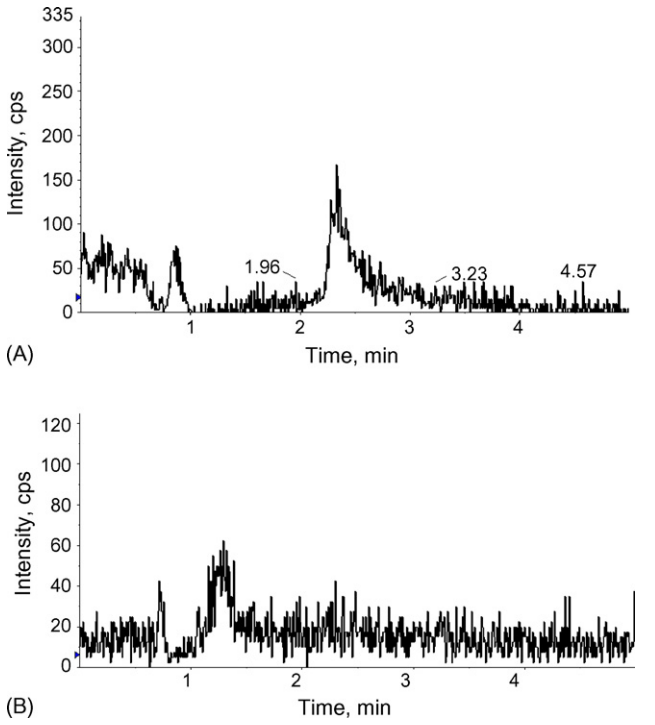

Fig. 5. Chromatograms obtained from a drug-free human plasma sample. Transitions for acquisition: (A) *m/z* 786.1 → 754.1 for cilnidipine; (B) *m/z* 268.3 → 116.1 for IS.

3.4. Stability

Since all samples were processed well within 6 h after thawing, we chose to leave QC samples at room temperature for 0, 2 and 6 h to investigate their stability. The calculated concentrations and R.S.D.% were listed in Table 3. The average accuracy ranged from 93.6 to 109% after being placed at room temperature for 2 h and the value was 101–109% for 6 h. These data

Table 1
Mean inter-day back-calculated concentrations and correlation coefficiencies

Nominal concentration (ng/ml)	Inter-day back calculated concentrations							*r*
	0.5	1.0	2.0	4.0	6.0	8.0	10	
Run ID								
I	0.54	0.88	2.03	4.17	5.81	8.08	9.99	0.9992
II	0.57	0.93	1.87	3.89	6.02	8.10	10.10	0.9993
III	0.56	0.96	1.91	3.89	5.73	7.93	10.50	0.9986
IV	0.58	0.88	1.91	3.97	6.06	8.05	10.10	0.9992
V	0.56	0.97	1.83	3.91	5.83	8.00	10.40	0.9989
Mean	0.56	0.93	1.91	3.97	5.89	8.03	10.21	0.9990
R.S.D. (%)	2.3	4.7	3.9	3.0	2.4	0.85	2.2	0.03
Accuracy (%)	112	92.5	95.5	99.2	98.2	100	102	

Table 2
Intra-batch and between-batch precisions and accuracy of rifaximin in human plasma

Nominal concentration (ng/ml)	Intra-batch ($n = 5$)		Between-batch ($n = 3$)	
	Measured concentration (ng/ml)	R.S.D. (%)	Measured concentration (ng/ml)	R.S.D. (%)
1	1.08 ± 0.03	3.0	0.98 ± 0.09	9.1
4	4.05 ± 0.16	3.9	3.98 ± 0.33	8.3
8	8.72 ± 0.19	2.2	8.65 ± 0.51	5.7

Table 3
Stability of rifaximin in plasma at room temperature

Nominal concentration (ng/ml)	0 h		2 h		6 h	
	Measured concentration (ng/ml) (mean ± S.D.)	R.S.D. (n = 3, %)	Measured concentration (ng/ml) (mean ± S.D.)	R.S.D. (n = 3, %)	Measured concentration (ng/ml) (mean ± S.D.)	R.S.D. (n = 3, %)
1	1.12 ± 0.04	3.6	1.09 ± 0.04	3.7	1.09 ± 0.04	3.7
4	3.97 ± 0.19	4.8	3.78 ± 0.02	0.5	4.08 ± 0.09	2.2
8	7.65 ± 0.24	3.1	7.49 ± 0.29	3.9	8.12 ± 0.13	1.6

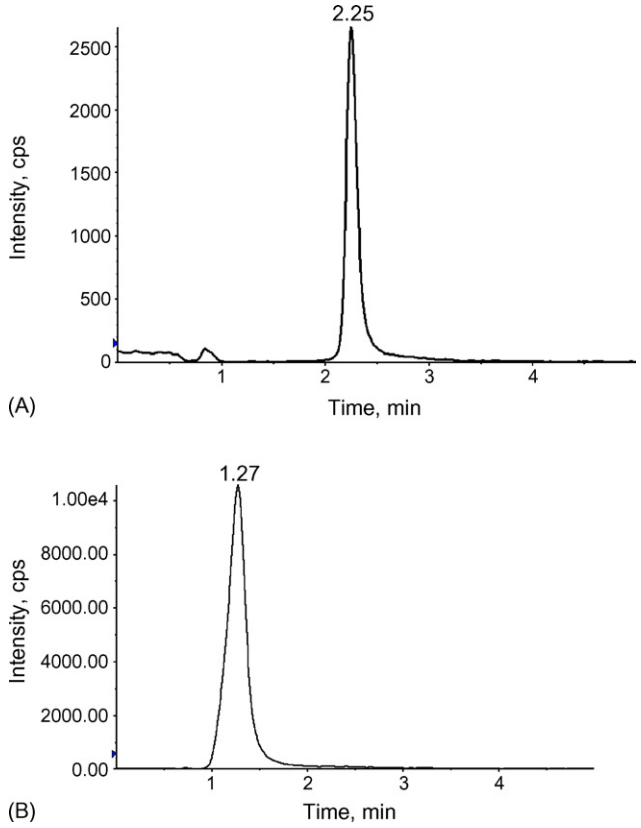

Fig. 6. Chromatograms obtained from a plasma sample of a patient (A) rifaximin (B) the IS.

showed that the spiked samples were stable after being exposed to room temperature for at least 6 h.

The freeze–thaw stability was also examined in the experiment. QC samples were subjected to freeze–thaw cycles consisting of a thaw to room temperature (more than 4 h), vortexing, and then refreezing (−40 °C) at least overnight. After each freeze–thaw cycle, the samples were analyzed and the calculated concentrations were summarized in Table 4. The accuracy was between 103 and 119% after 1 cycle and between 103 and 117% after three cycles. These results indicated that the samples were stable after three freeze–thaw cycles.

3.5. Analysis of clinical samples

The blood samples from 10 clinical HE patients who were treated with rifaximin orally at a dose of 200 mg were obtained and the plasma concentrations of rifaximin were determined with the developed method. This is not a full course pharmacokinetic study and blood samples were collected only to 4 h after drug administration. As shown in Table 5, rifaximin was not detected in most patients in the early hours of drug administration, agreeing with the non-absorption characteristic of this drug. But at 4 h some patients showed very low levels of rifaximin in their blood. In general, our results were consistent with those reported in the literature [7], demonstrating the concentration of rifaximin in plasma was undetectable in most samples. Our results confirmed the low absorption of this drug and its passage into general circulation was negligible in most patients. However, because the sensitivity and selectivity of this method

Table 5
Plasma concentration of rifaximin from 10 clinical patients

Patient number	Plasma concentration (ng/ml)			
	0 h	1 h	2 h	4 h
1	nd	nd	nd	nd
2	nd	nd	nd	0.66
3	nd	nd	nd	nd
4	nd	nd	nd	0.52
5	nd	nd	nd	0.60
6	nd	nd	nd	nd
7	nd	nd	nd	0.72
8	nd	nd	nd	nd
9	nd	nd	nd	nd
10	nd	nd	nd	nd

nd: not detected.

Table 4
Freeze–thaw stability of plasma samples

Nominal concentration (ng/ml)	Before freeze		After 1 freeze–thaw cycle		After 3 freeze–thaw cycles	
	Measured concentration (ng/ml) (mean ± S.D)	R.S.D. (n = 3, %)	Measured concentration (ng/ml) (mean ± S.D)	R.S.D. (n = 3, %)	Measured concentration (ng/ml) (mean ± S.D)	R.S.D. (n = 3, %)
1	1.13 ± 0.02	2.2	1.19 ± 0.01	0.5	1.17 ± 0.02	1.3
4	4.28 ± 0.20	4.6	4.53 ± 0.06	1.2	4.57 ± 0.01	0.1
8	8.17 ± 0.38	4.6	8.27 ± 0.32	3.9	8.26 ± 0.32	3.9

were much higher than those used in the literature, we did find traces of rifaximin after 4 h of administration in 40% of the patients. Moreover, the plasma used in this assay was only 0.2 ml, about 1/5 of that used in the literature. The small sample volume required by our method would alleviate the discomfort of participating patients.

It would be interesting to correlate blood levels of rifaximin with clinical observations of the treated patients. The presence or absence of rifaximin in blood may provide some insights into the mechanism of actions of the drug in the treatment of HE patients.

4. Conclusion

An LC–MS/MS method was developed and validated for the quantification of rifaximin in human plasma. This method is rapid, selective and highly sensitive with a quantification limit of 0.5 ng/ml. Only 0.2 ml of plasma is needed for analysis with this method, which greatly facilitated the collection of samples. The method allows quantification of rifaximin over the range 0.5–10 ng/ml. The analysis time was decreased comparing to conventional HPLC methods. For each run, only 5 min was needed. This method was successfully applied to evaluate the plasma concentration of rifaximin in patients. It provides a practical tool for *in vivo* detection of rifaximin.

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