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Quantitative determination of erythromycylamine in human plasma by liquid chromatography–mass spectrometry and its application in a bioequivalence study of dirithromycin

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Abstract

A sensitive, rapid liquid chromatographic–electrospray ionization mass spectrometric method for determination of erythromycylamine in human plasma was developed and validated. Erythromycylamine in plasma (0.2 mL) was extracted with ethyl acetate, the organic phase was transferred to another clear 1.5 mL Eppendorf tube and evaporated to dryness under gentle nitrogen stream at 45 °C, and the residue was dissolved in 100 μL of mobile phase. The samples were separated using a Thermo Hypersil HyPURITY C18 reversed-phase column (150 mm × 2.1 mm I.D., 5 μm). A mobile phase containing 10 mM of ammonium acetate (pH = 6.4)-acetonitrile-methanol (50:10:40, v/v/v) was used isocratically eluting at a flow rate of 0.2 mL/min. Erythromycylamine and its internal standard (IS), midecamycin, were measured by electrospray ion source in positive selective ion monitoring mode. The method demonstrated that good linearity ranged from 4.5 to 720 ng/mL with $r = 0.9997$. The limit of quantification for erythromycylamine in plasma was 4.5 ng/mL with good accuracy and precision. The mean extraction recovery of the method was higher than 75.1% and 72.7% for erythromycylamine and IS, respectively. The intra-day and inter-day precision ranged from 5.2% to 6.4% and 5.6–9.3% (relative standard deviation, RSD), respectively. The established method has been successfully applied to a bioequivalence study of two dirithromycin formulations for 18 healthy volunteers.

Keywords: Dirithromycin; Erythromycylamine; LC–MS; Electrospray ionization; Bioequivalence

1. Introduction

Dirithromycin is a semisynthetic derivative of erythromycin, a 14-membered ring macrolide antibiotic. The drug is rapidly and nonenzymatically hydrolyzed during absorption and distribution, to its active metabolite 9-(S)-erythromycylamine which is the predominant compound found in plasma and extravascular tissues. High tissue concentration of erythromycylamine is achieved after oral doses of dirithromycin, erythromycylamine slowly releases back into the circulation [1]. The antimicrobial spectrum of erythromycylamine is similar to that of erythromycin. The mechanism of action of erythromycylamine is like that of erythromycin and other macrolides. Its long half-life, high tissue concentrations and good tolerance makes it an interesting alternative in the treatment of appropriate community-acquired infections, notably of respiratory-tract infections [2–13].

So far, the reports about bioavailability and bioequivalence studies and method for the determination of dirithromycin (erythromycylamine) in biological sample are very few. The development of a sensitive method for determination of erythromycylamine in human plasma is of importance for the investigation of bioavailability and bioequivalence of this compound. Generally, the determination of antibiotics, including macrolide antibiotics, is mainly carried out by microbiological assays in bioavailability and bioequivalence study [14–16]. However, the bioassays are lack of specificity and it is difficult to confirm which drugs remained in biological sample. To solve

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the problems, chemical analyses such as high-performance liquid chromatographic (HPLC) techniques with electrochemical detection [17,18] was used for the determination of erythromycylamine in biological sample. However, the reported methods have less sensitivity and specificity. Highly sensitive and selective methods without time-consuming sample pretreatment are desired for bioavailability and bioequivalence studies. Liquid chromatography–mass spectrometry (LC–MS) is the most promising technique for separation and quantitative analysis of drugs. The aim of the work is to develop and validate a simple, highly sensitive and selective LC–MS method for quantitative analysis of erythromycylamine in human plasma. The established method was applied to the bioequivalence study.

2. Experimental

2.1. Reagents and chemicals

Erythromycylamine (purity > 99.5%) reference standard and midecamycin (internal standard, IS, purity > 98.9%) were purchased from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Acetonitrile was supplied by Caledon Laboratories Ltd. (Georgetown, Ont., Canada). HPLC grade methanol was obtained from TEDIA company, Inc. (Fairfield, OH, USA). Other reagents were of analytical grade, and all water used was Milli-Q grade.

2.2. Equipment

The HPLC system consisted of a Shimadzu LC-10Advp pump, an SCL-10Advp system controller, a CTO-10Avp column oven, an FCV-10ALvp low-pressure gradient unit, a DGU-14A degasser (Shimadzu, Kyoto, Japan). The mass spectrometer was an LCMS-2010 single quadrupole equipped with electrospray ionization interface (Shimadzu, Kyoto, Japan). The data were collected and processed using LCMSsolution software.

2.3. Chromatographic conditions

Chromatographic separations were performed using a Thermo Hypersil HyPURITY C18 (150 mm × 2.1 mm, 5 μm) analytical column. The oven temperature was set at 45 °C. The mobile phase containing 10 mM ammonium acetate (adjusted pH to 6.40 with acetic acid)-acetonitrile-methanol (50:10:40, v/v/v) was used at a flow rate of 0.2 mL/min.

2.4. Mass spectrometer conditions

An LCMS-2010 quadrupole mass spectrometer was interfaced with electrospray ionization (ESI) probe. The temperatures were maintained at 250 °C, 250 °C and 200 °C for the probe, CDL and block, respectively. The voltages were set at 4.5 kV, −30 V, 25 V, 150 V and 1.6 kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array RF and detector, respectively. The flow rate of nebulizer gas and dried gas were set at 1.5 and 10 L/min, respectively. The ions of selection monitoring were decided by positive scanning from *m/z* 100–1000. For the quantification of erythromycylamine, the positive protonated molecule ions of erythromycylamine at $m/z = 368.3$ $[M + 2H]^{2+}$ and midecamycin (IS) at $m/z = 814.7$ $[M + H]^{+}$ were monitored. Tuning of mass spectrometer was performed with the help of autotuning function of LCMSsolution software (Version 2.02) using tuning standard solution (polypropylene glycol). Optimization and calibration of mass spectrometer were achieved with autotuning.

2.5. Analytical procedure

2.5.1. Preparation of stock solutions, calibration standard and quality control samples

A stock solution of erythromycylamine in methanol at concentration of 100 μg/mL was prepared. The internal standard (midecamycin) was also prepared as a stock solution (125 μg/mL) in methanol and was further diluted with methanol to 800 ng/mL and used for all analyses. A serial calibration curve samples at concentrations of 4.5, 9.0, 18.0, 36.0, 72.0, 140.0, 360 and 720 ng/mL of erythromycylamine were freshly prepared by serially diluting stock solution with drug-free plasma. Firstly, plasma sample containing 720 ng/mL of erythromycylamine was prepared by spiking with erythromycylamine stock solution to drug-free plasma. Then the prepared plasma sample containing 720 ng/mL of erythromycylamine was serially diluted with drug-free plasma to form calibration samples. The quality control (QC) samples were separately prepared by adding standard solution to drug-free plasma at concentrations of 4.5 (LOQ), 9.0 (low), 72.0 (medium) and 360.0 ng/mL (high) of erythromycylamine. All plasma samples were stored at −20 °C. Fifty micro liters of IS (800 ng/mL) was added to 0.2 mL of calibration curve samples and QC samples, respectively. The further processing of both calibration curve samples and QC samples were the same as described in Section 2.5.2 for collection and preparation of the samples. All standard stock solutions were prepared once a month and stored at −20 °C.

2.5.2. Collection and preparation of the samples

Eighteen healthy male volunteers received the investigation. The average age of volunteers was 25.6 years old within the range of 20–31. The mean of body weights was 65.9.4 kg (61–75.2 kg) and the mean of body heights was 169.5 cm (165–177 cm). Subjects were included based on their medical history, clinical examination results and routine laboratory test results. All eligible subjects provided written informed consent for participation in the study. A 2 × 2, crossover, randomized, open-label design was used. Subjects were randomly assigned to receive reference formulation followed by test formulation with a 2-week washout period between doses. After a 12-h (overnight) fast, subjects received a single, 500-mg oral dose (tablet) of dirithromycin with 200 mL of water. Blood samples were collected in heparinized tubes pre-dose (0 h) and at 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 10, 24, 48, 72, 96, 120 and 144 h post-dose. Plasma was immediately separated by centrifugation at 4000 rpm and stored at −20 °C until analysis. A plasma sample (0.2 mL) was placed in a 2 mL Eppendorf tube. After the addition of 50 μL of 800 ng/mL solution of IS and 100 μL of

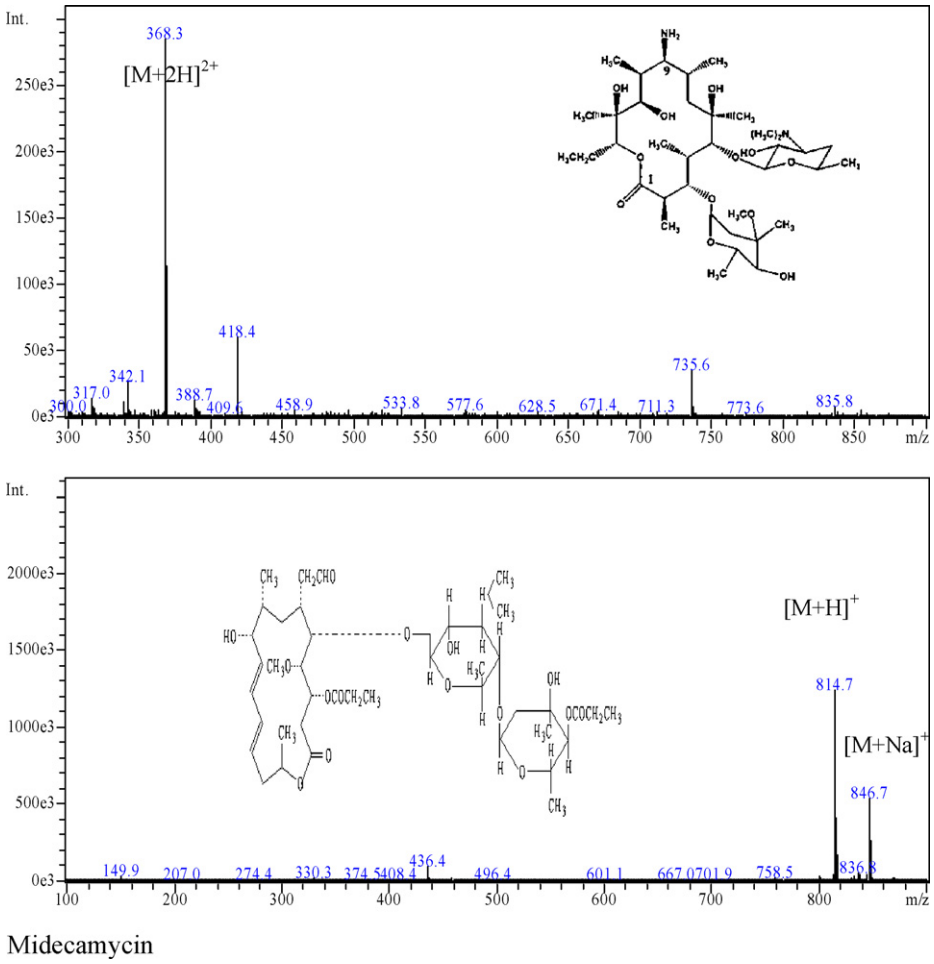

Fig. 1. ESI-MS positive ion scanning spectra of erythromycylamine and midecamycin (IS). Chromatographic conditions: column: Thermo Hypersil HyPURITY C18 (150 mm × 2.1 mm, 5 μm), oven temperature: 45 °C, mobile phase: 10 mM ammonium acetate (adjusted pH to 6.4 with acetic acid)-acetonitrile-methanol (50:10:40, v/v), flow rate: 0.2 mL/min. Mass spectrometer: electrospray ionization (ESI) source, positive scan *m/z*: 100–1000, temperature: probe: 250 °C, CDL: 250 °C, block: 200 °C, voltage: capillary: 4.5 kV, CDL: −30 V, Q-array 1, 2, 3 bias: 25 V, Q-array RF: 150 V, detector: 1.5 kV. Nebulizer gas flow rate: 1.5 L/min.

solution of saturated sodium carbonate, the tube was briefly vortexed and 1 mL of ethyl acetate was added into the tube. After vortexing for 3 min, the tube was centrifuged at 14,000 rpm for 5 min at room temperature and the organic phase was transferred to another clear 1.5 mL Eppendorf tube. The extract was evaporated to dryness under gentle nitrogen stream at 45 °C. The residue was redissolved in 100 μL of mobile phase. The tube was vortexed for 30 s and centrifuged at 14,000 rpm for 5 min, and 5 μL of supernatant was injected onto the analytical column.

3. Results and discussion

3.1. Mass spectrometry

Erythromycylamine consists of a 14-membered macrocyclic lactone ring onto which two sugar moieties are linked. An aminosugar, D-desosamine, is attached through a β-glycosidic bond to the C5 position of the lactone ring. A neutral sugar, L-cladinose, is attached via an α-glycosidic linkage to the C3 position of the lactone ring. The molecule of erythromycylamine contains two amino groups, one is linked to C9 of the 14-membered ring, and another attaches to the sugar. These two amino groups were simultaneously protonated easily during ionization process in the acidic condition. In the present study, erythromycylamine and midecamycin (IS) were scanned with ESI and APCI positive and negative ion modes using injection standard solutions. In different ionization modes, the base peak intensities of positive ion were higher than those of negative ion, and the efficiency of ionization in ESI was higher than APCI. The protonated molecules with an *m/z* 368.3 $[M+2H]^{2+}$ and *m/z* 735.6 $[M+H]^{+}$ for erythromycylamine were produced. The ion *m/z* 368.3 was more sensitive than *m/z* 735.6. The ion $[M+2H]^{2+}$ with *m/z* 368.3 was observed in the article of González de la Huebra et al. [19], but this ion was not explained. Midecamycin (IS) molecule was protonated to form molecule ion with *m/z* 814.7 $[M+H]^{+}$ because of one nitrogen in the molecule. Fig. 1 shows the positive ion mass spectra of erythromycylamine and midecamycin (IS) by ESI scanning from *m/z* 100 to 1000. Selected ion monitoring was chosen for the method since its sensitivity and selectivity.

3.2. Selection of HPLC conditions

Erythromycylamine is a lipophilic, with basic property compound. Two different types of column (Thermo Hypersil HyPURITY C18 and Shim-Pack ODS) were used to obtain optimized response, suitable retention time and good peak shapes for erythromycylamine and midecamycin. The Thermo Hypersil HyPURITY C18 column was selected for all analysis since it provided symmetrical peak shape and obtained the highest ion intensity to erythromycylamine. The separation and ionization of erythromycylamine and midecamycin were affected by composition of mobile phase. The mobile phase pH affected not only the retention time, but also the ionization efficiency of erythromycylamine and midecamycin. The retention time was prolonged by the increasing of mobile phase pH, especially erythromycylamine. The acidity of mobile phase benefited to the ionization of erythromycylamine and midecamycin. Thus the sensitivity of erythromycylamine was improved by increasing acidity of mobile phase because of raising the ionization efficiency. Diana et al. [20] separated dirithromycin and related substances using mobile phase with pH 7.5. In this study, the mobile phase with pH 6.4 was applied to eluting for increasing the sensitivity of erythromycylamine. Raising the concentration of ammonium acetate in mobile phase can prolong the retention time of analyte under the same organic solvent percentage. Thus, increasing the concentration of ammonium acetate in mobile phase resulted in the higher percentage of organic solvent to maintain the same retention time for erythromycylamine and midecamycin. Increasing the proportion of methanol can increase the ionization efficiency of erythromycylamine and the peak shape was improved by acetonitrile.

3.3. Validation of the method

3.3.1. Matrix effect and extraction recovery

Three sets of eight calibration standards and blank spiked with IS were prepared for evaluation of recovery and ionization suppression or enhancement. The standard solutions were diluted with mobile phase to reach the concentration of 4.5, 9.0, 18.0, 36.0, 72.0, 140.0, 360 and 720 ng/mL in set 1. Set 2 consisted of eight plasma samples spiked with standard solutions after extraction to the same concentrations series as set 1. Plasma samples spiked with standard solution before extraction and S0 (blank plasma spiked with IS) were processed and analyzed to obtain set 3. Five replicates of each set were used for determination of recovery and absolute matrix effect (ME). Internal standard was not added to standards. The matrix effect, i.e. the possibility of suppression or enhancement of ionization and the effect of the matrix on recovery (RE) was evaluated by comparing results from analysis of three sets of samples as follows:

$$\mathrm{ME}\,(\%) = \frac{B}{A} \times 100$$

$$\mathrm{RE}\,(\%) = \frac{C}{A} \times 100$$

where A is the peak area of set 1, B the peak area of set 2 and C is the peak area of set 3.

By comparing peak areas of standard and IS of samples spiked with standards after extraction with the analogous peak areas obtained by injecting neat standard and IS directly, the extent of the absolute ME was estimated (Table 1). The values >100% indicated ionization enhancement in plasma versus neat standards, whereas values <100% indicated ionization suppression. The data presented in Table 1 indicated that the mean "absolute" ME for erythromycylamine and midecamycin (99.6% and 101.4%, respectively) was negligible. In addition, the coefficients of variation (CV's, %) of the mean peak areas of erythromycylamine at eight calibration standard concentrations and midecamycin in five different plasma lots were small (<11%, Table 2), strongly indicating little or no difference in ionization efficiency and consistent recovery of standard and IS from different plasma lots. Direct precipitation protein with acetonitrile, perchloric acid or trichloroacetic acid was used for processing plasma samples and for obtaining low recovery. Various liquid–liquid extraction methods were investigated for the extraction of erythromycylamine from plasma. By the comparison of extraction efficiency of different organic solvents including diethyl ether, ethyl acetate, methyl *tert*-butyl ether, hexane and dichloromethane, the result showed that ethyl acetate gave the high recovery (75.1% and 72.7%) for the erythromycylamine and the IS (Table 1). The other organic solvents used gave lower extraction recovery (data was not shown). The pH of plasma sample was adjusted to pH 10–11 with the sat-

Table 1
Recovery and matrix effects of erythromycylamine and midecamycin extraction from human plasma

Nominal concentration (ng mL^{-1})	Mean peak area ($n = 5$)			ME (%)	RE (%)
	Set 1	Set 2	Set 3		
4.5	49,273	48,924	35,687	99.3	72.4
9	97,273	97,532	68,637	100.3	70.6
18	186,343	192,713	151,823	103.4	81.5
36	372,754	369,935	293,748	99.2	78.8
72	836,497	824,325	589,696	95.9	70.5
140	1,627,632	1,659,874	1,212,387	98.5	74.5
360	4,649,742	4,562,832	3,325,269	102.0	71.5
720	9,256,785	9,387,362	6,727,465	98.1	72.7
S0 (IS)	238,233	228,765	169,078	101.4	80.0

Table 2
Intermediate precision, accuracy and linear regression parameters of erythromycylamine determination in human plasma

Added concentration (ng/mL)	Mean measured concentration ($n = 5$) (ng/mL)	Precision (RSD, %)	Mean relative error[a] (%)
4.5	4.4	10.6	2.2
9.0	8.7	6.5	3.3
18.0	17.2	5.6	4.4
36.0	35.2	6.3	2.2
72.0	68.7	5.4	4.6
140.0	132.8	4.2	5.1
360.0	348.3	5.3	3.3
720.0	730.4	5.1	1.4

Calibration curve:

Slope	0.014
Intercept	−0.0214
Correlation coefficient	0.9997

[a] Mean relative error = |mean measured concentration − added concentration| × 100/added concentration.

Table 3
Reproducibility and accuracy for erythromycylamine of quality control sample in human plasma ($n = 5$)

Nominal concentration (ng/mL)	Mean found concentration (ng/mL)	Precision (RSD, %)	Mean relative error[a] (%)
Intra-day			
4.5	4.6	7.9	2.2
9	8.9	6.4	1.1
72	71.8	6.3	0.3
360	355.1	5.2	1.4
Inter-day			
4.5	4.3	11.4	4.4
9	9.1	9.2	1.1
72	73.0	9.3	1.4
360	358.0	5.6	0.6

[a] Mean relative error = |mean measured concentration − added concentration| × 100/added concentration.

urated solution of sodium carbonate during extraction. In our study, the recoveries of erythromycylamine and IS were calculated by comparing the areas obtained from spiked blank plasma ($n = 5$) with those obtained from injecting directly standard solutions with the same concentrations in mobile phase. The mean extraction recoveries were higher than 75.1% and 72.7% for erythromycylamine and IS, respectively.

3.3.2. Selectivity

Six lots of blank plasma extracts from different sources were analyzed. Interference peaks from endogenous substances in free-drug human plasma at the retention time of erythromycylamine and midecamycin were not observed in any of the plasma lots. In addition, erythromycylamine and midecamycin were separately injected and selective ions were monitored. Fig. 2(A) showed one of the representative chromatogram of six lots of blank plasma extracts. Fig. 2(B) showed the selective ion chromatogram of the plasma sample at the lowest limit of detection (LLOD) (1.0 ng/mL). No cross-talk was observed.

3.3.3. Sensitivity and linearity

The limit of quantitation (LOQ) using 0.2 mL plasma with acceptable accuracy and precision (<15%) is 4.5 ng/mL. A good signal-to-noise ratio was observed at the LOQ indicating that the corresponding value could be reached. The LLOD was estimated as the amount of erythromycylamine that gave a signal three times the noise (S/N ≥ 3); it was calculated to be 1.0 ng/mL. The

Table 4
Pharmacokinetic properties of two oral formulations of single-dose dirithromycin of 500 mg in healthy subjects ($n = 18$)

Property	Test formulation (*T*)	Reference formulation (*R*)	*T*/*R*
C_{max} (ng/mL)	347.9 (138)	352.3 (121)	0.99 (0.2)
T_{max}, h	2.61 (0.7)	2.50 (0.7)	1.04 (0.3)
AUC_{0-t} (ng/(mL h))	4333.3 (1432)	4259.0 (1333)	1.07 (0.3)
$AUC_{0-\infty}$ (ng/(mL h))	4871.6 (1277)	4790.2 (1258)	1.06 (0.3)
$T_{1/2}$	44.8 (10)	43.7 (9)	

* Values are mean (SD)

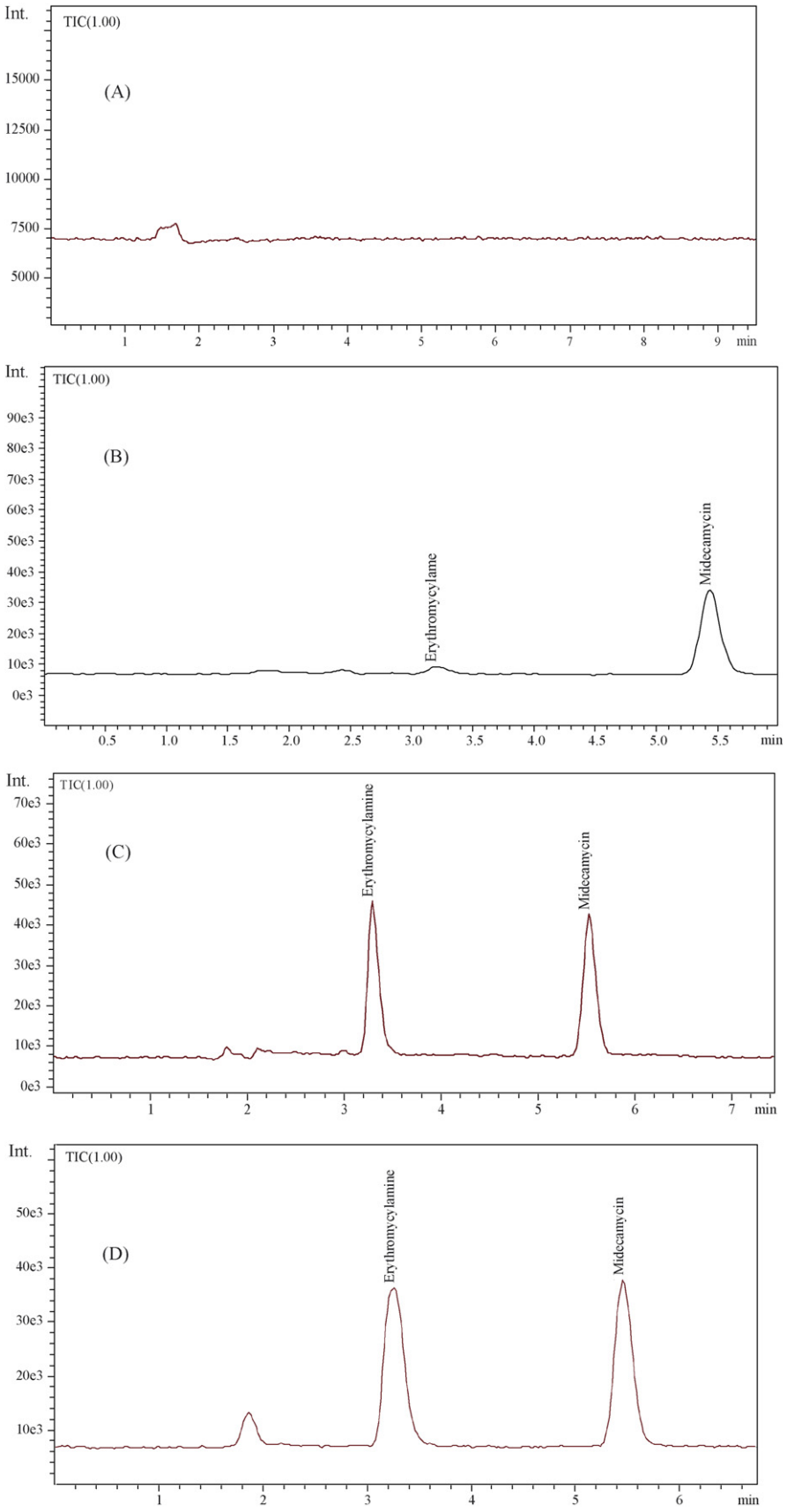

Fig. 2. Selective ion chromatograms of erythromycylamine and midecamycin (IS). Positive ion monitored at *m/z* 368.3 (erythromycylamine), 814.7 (midecamycin, IS), (A) blank plasma, (B) blank plasma spiked with 1.0 ng/mL (the lowest limit of detection) of erythromycylamine and IS, (C) blank plasma spiked with standard (100 ng/mL) and IS, and (D) human plasma sample after administration of erythromycylamine and spiked with IS.

eight-point calibration plots obtained by weighted linear regression were highly linear over the range from 4.5 to 720 ng/mL with the correlation coefficient of 0.9997. The calibration curve had the regression equation of $y = 0.014x - 0.0214$, where y was the peak area ratio of erythromycylamine to IS, x was the concentration of erythromycylamine. Representative calibration curve parameters for the method from intra-day standard curve replicates are shown in Table 2. Intra-assay precision and accuracy were very satisfactory for all the concentrations tested. relative standard deviation (RSD) values were less than 10.6% at all concentrations.

3.3.4. Repeatability and accuracy

The intra-day and inter-day repeatability of the method for plasma is summarized in Table 3 by analysis of replicates ($n = 5$) of LOQ and QC samples containing known concentrations of 4.5, 9, 72 and 360 ng/mL of erythromycylamine. The precision of the method was described as RSD among each assay. The intra-day RSDs were always below 7.9% and the inter-day RSDs were within 11.4%. The accuracy of the method was evaluated by analysis of the QC samples spiked with standard solutions and expressed as a percentage error of measured concentrations versus nominal concentrations. Precision and accuracy were calculated at each concentration. The results of the precision and accuracy of the proposed method were acceptable for bioequivalence.

3.3.5. Stability

The stability of erythromycylamine and IS in human plasma under different storage conditions was evaluated as follows: QC samples were subjected to short-term room temperature conditions, to four freeze-thaw cycles stability studies, to long-term (2-month) storage conditions (−20 °C) and to processed sample kept at room temperature. All the stability studies were conducted at three concentration levels (9, 72 and 360 ng/ml) with five determinations for each. For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time (around 24 h) during routine sample preparation. Samples were extracted and analyzed as described above. These results indicated reliable stability behavior under the experimental conditions of the regular analytical procedure. Erythromycylamine is stable at room temperature for at least 24 h. The analyte is also stable in human plasma when stored at −20 °C for at least 2 months and at room temperature for at least 24 h. It is also stable under the influence of four freeze-thaw cycles.

3.4. Bioequivalence study

The developed and validated method has been successfully employed to determine erythromycylamine concentrations in human plasma samples after the administration of a 500 mg oral dose of dirithromycin. Table 4 showed the pharmacokinetic parameters of test and reference formulations. In this study in healthy volunteers, a single, 500-mg dose of test formulation was found to be bioequivalent to reference formulation based on the rate and extent of absorption. Representative concentration versus time profile for a subject, receiving a single dose, is presented in Fig. 3.

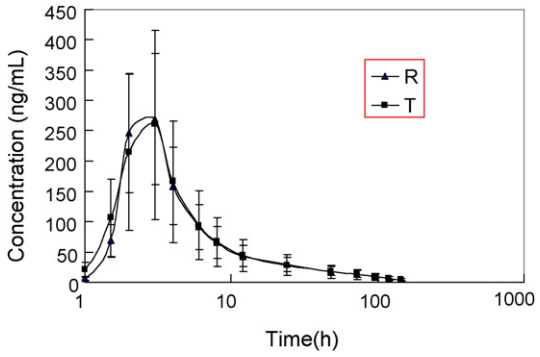

Fig. 3. Mean plasma concentration–time profile of erythromycylamine from 18 healthy volunteers following a single oral dose of 500 mg. *T*—test formulation; *R*—reference formulation.

4. Conclusion

A sensitive, rapid and specific LC–MS method has been described for the determination of erythromycylamine in human plasma. The appropriate mobile phase was used and double charged ion $[M + 2H]^{2+}$ for erythromycylamine was selectively monitored. The lower limit of quantification (4.5 ng/mL) using 0.2 mL of plasma and a simple procedure of pretreatment were obtained. The method has been successfully applied to the bioequivalence studies and demonstrated that the method is reproducible. The pharmacokinetic parameters after a single 500-mg dose of dirithromycin in healthy volunteers were obtained.

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