Nanjing Medical University[1], Jiangsu Provincial Institute of Materia Medica[2], Nanjing, China

Determination of azithromycin in human plasma by LC-MS-MS and its pharmacokinetics

Zhou Xue-Min[1], Liu Jie[1], Gao Juan[1], Yao Quan-Sheng[2], Wang Wen-Yan[2]

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Zhou Xue Min, Nanjing Medical University, School of Pharmacy, Hanzhong Road 140, Nanjing City, PO Box 210029, China

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A LC-MS-MS assay has been developed for determinating of azithromycin in human plasma and investigating the pharmacokinetics in Chinese male volunteers following oral administration of a single dose of the capsules (0.5 g). Plasma samples were deproteinated by methanol and a liquid chromatographic-mass-mass spectrometric assay was developed for the determination of azithromycin in human plasma. Assay linearity was obtained in the range of 3.048–1016 $\mu g \cdot L^{-1}$ ($r = 0.9995$). The recovery of azithromycin from human plasma was more than 90%. The intra- and inter-day precision for four different concentration examined were lower than 15%. Its main pharmacokinetic parameters of AUC_{0-144}, $AUC_{0-\infty}$, C_{max}, T_{max}, $T_{1/2}$ and MRT were determined from plasma concentrations for both formulations and found to be in good agreement with the reported values. AUC_{0-144}, T_{max} and C_{max} were tested for bioequivalence after log-transformation of data. No significant difference was found based on ANOVA. The test formulation was bioequivalent to the reference formulation and there was no significant difference of T_{max} between the test formulation and reference formulation.

1. Introduction

Azithromycin is the prototype of a subclass of macrolide antibiotics known as the azalides (Bright et al. 1988). It differs structurally from erythromycin by insertion of a methyl-substituted nitrogen at position 9a the lacton ring, creating a 15-membered macrolide (Shepard 1990).

The purpose of this study was to compare the bioavailability (rate and extent of absorption) of genetic formulations of the test product (azithromycin capsule) relative to the reference formulation (Azithromycin Capsules). The bioequivalence of the test formulation was assessed by statisitical analysis of the pharmacokinetic parameters as recommended by the China Pharmaceutical University.

2. Investigations and results

Under the conditions described in the experimental part, the assay was highly specific, and no endogenous plasma materials interfered with the peak of azithromycin or roxithromycin (Fig. 1). Azithromycin and roxithromycin were eluted with retention times of 1.8 min and 5.2 min, respectively. The calibration cure was linear from 3.048 to 1016 $\mu g \cdot L^{-1}$ ($f = 5.936 \times 10^{-3}\,C - 4.131 \times 10^{-4}$, $r = 0.9995$). The RSD of intraday precision were 5.9%, 3.9%, 3.0% and 2.1% respectively in four different concentrations from low to high. The RSD of interday precision were 7.4%, 11.2%, 4.4% and 3.6%, respectively, in four different concentrations from low to high (Table 1). The recovery of azithromycin from human plasma was more than 90% (Table 2) and the data was $104.2 \pm 7.3\%$, $107.3 \pm 3.9\%$, $97.64 \pm 1.2\%$ and $94.86 \pm 5.7\%$, respectively, in four different concentrations from low to high. The stability of azithromycin in plasma was fine (Table 3) including the stability for 24 h at ordinary temperature (3.533 ng/ml, 30.24 ng/ml, 203.9 ng/ml and 1013 ng/ml, respectively) and the stability of 50 days when frozen (3.146 ng/ml, 32.39 ng/ml, 197.0 ng/ml and 1096 ng/ml, respectively). The low concentration of quality control samples was in the range of 80%–120% (3.505 ± 0.101 ng/ml), and the others were all in the range of 85%–115% (29.69 ± 2.44 ng/ml, 203.9 ± 6.7 ng/ml and 1035 ± 67 ng/ml, respectively) (Table 4). The C_{max} and AUC_{0-144} were log-transformated then calculated on ANOVA. The 90% confidence intervals were 84.53%–122.31% for C_{max} and 93.86%–110.60% for AUC_{0-144} respectively. A Wilcoxon rank sum test was done for T_{max} and the result was $P > 0.05$. No significant difference was found.

In the present work, azithromycin was well tolerated by the subjects; unexpected incidents that could have influenced the outcome of the study did not occur. All volunteers who started the study continued to the end and were discharged in good health. Both formulations were readily absorbed from the gastrointestinal tract and azithromycin was measurable at the first sampling time in almost all volunteers. The mean concentration-time profiles for the two formulations of are shown in Fig. 2. All calculated pharmacokinetic parameter values agreed well with the previously reported values. The relative bioavailability of the test formulation was $103.9 \pm 21.0\%$. Table 5 shows the main pharmacokinetic parameter of the two formula-

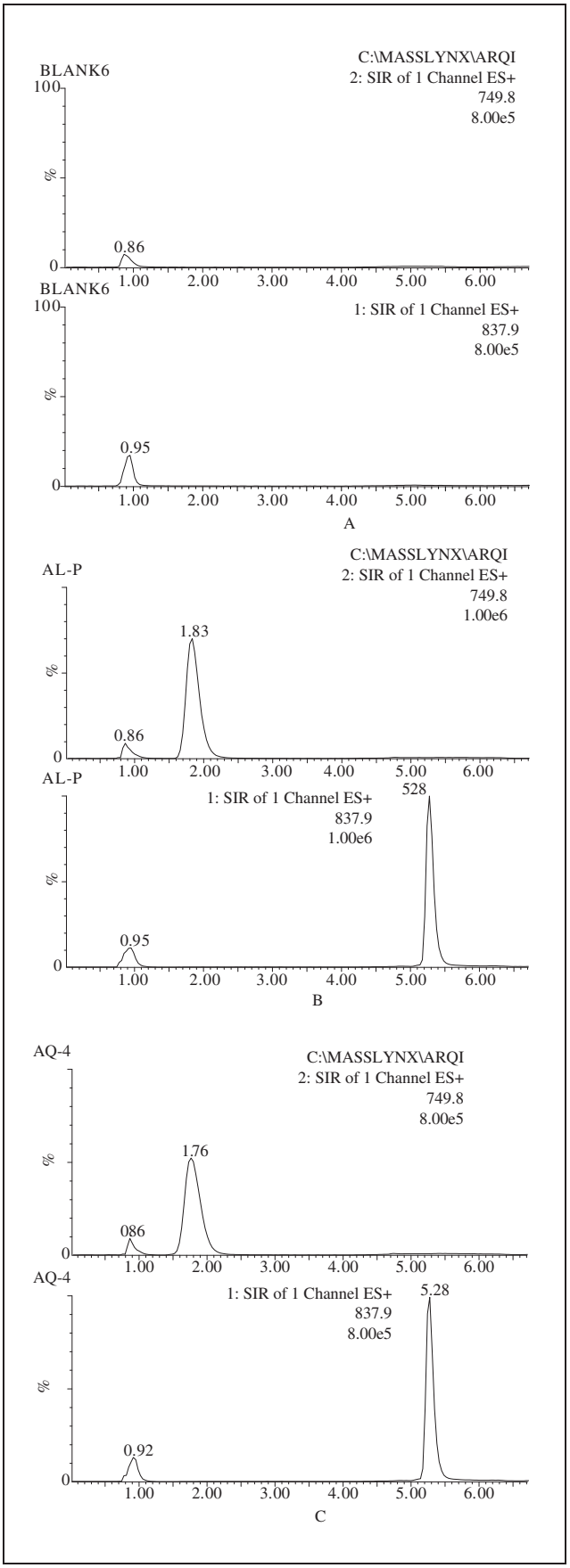

Fig. 1: Chromatograms of azithromycin using LC-MS-MS; A) Blank plasma; B) Blank plasma spiked with azithromycin and the internal standard; C) Plasma sample after a single oral administration of azithromycin

Table 1: Interday and intraday precision of azithromycin in plasma

C (ng · ml^{-1})	Mean	RSD (intraday, %)	RSD (interday, %)
3.048	3.116	5.9	7.4
30.48	31.08	3.9	11.2
203.2	202.7	3.0	4.4
1016	1013	2.1	3.6

Table 2: Recovery of azithromycin in plasma

C (ng · ml^{-1})	Mean (%)	RSD %
3.048	104.2	7.02
30.48	107.3	3.66
203.2	97.64	1.21
1016	94.86	5.96

Table 3: Stability of azithromycin in plasma

C (ng·ml^{-1})		3.072	30.72	204.8	1024
Average of concentration (ng · ml^{-1})	24 h for ordinary temperature (n = 3)	3.533	30.24	203.9	1013
	50 days for freeze (n = 2)	3.146	32.39	197.0	1096

Table 4: The quality control samples

C (ng/ml)	1	2	3	4	the average of concentration	RSD %
3.072	3.607	3.575	3.418	3.418	3.505	2.87
30.72	28.99	28.42	33.31	28.05	29.69	8.23
204.8	196.5	212.7	235.0	202.5	203.9	3.27
1024	988.0	968.1	1084	1101	1035	6.46

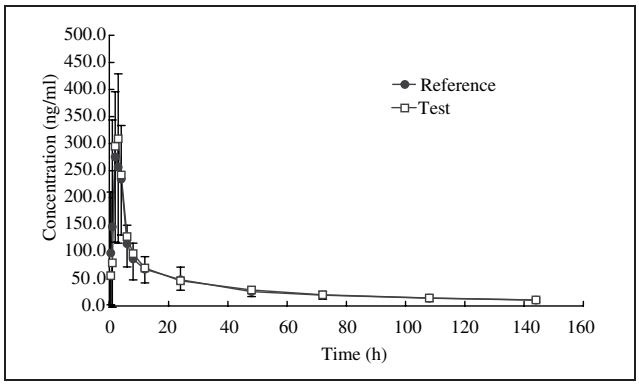

Fig. 2: Mean plasma concentration of azithromycin, 2 × 250 mg capsules, after oral administration of a single dose of the two brands to 24 healthy human volunteers

Table 5: Pharmacokinetic parameter of azithromycin formulations

Pharmacokinetic parameter	Test formulation	Preference formulation
AUC_{0-144} (ng/ml · h)	5062 ± 1778	4899 ± 1455
$AUC_{0-\infty}$ (ng/ml · h)	5676 ± 1988	5510 ± 1692
C_{max} (ng/ml)	426 ± 179	413 ± 167
T_{max} (h)	2.6 ± 0.8	2.5 ± 1.0
$T_{1/2}$ (h)	38.00 ± 4.42	38.28 ± 5.95
MRT	56.43 ± 5.98	56.65 ± 7.53
Percentage of extrapolated part of the AUC (%)	11 ± 2	11 ± 3

tions. Based on the pharmacokinetic and statistical results of this study, we can conclude that the two formulation were bioequivalent.

3. Discussion

There is no conjugated system in the structure of azithromycin, and with a HPLC-UV method it is hard to reach the required limit of quantification (LOQ). In the study presented here, we established a new analytical method using LC-MS-MS. The interference in the blank plasma chromatograph was greatly cut down in SIM mode, ie, m/z 749.8 for azithromycin and m/z 837.9 for roxithromycin, respectively. The LOQ of 3.048 ng/ml was achieved using this method, which was sensitive enough for determination of azithromycin concentration in human plasma. Moreover, the sample extraction procedure was quite simple and stable. In conclusion, the LC-MS-MS method described in this report was highly sensitive and specific enough for accurate determination of the plasma level of azithromycin.

4. Experimental

4.1. Materials

Test product: Azithromycin 250 mg capsule (dose 2 capsules = 500 mg). Batch NO.: 050402, Pharmaceutical Factory of Ba qiuen Medical University. Reference product: Azithromycin 250 mg capsule (dose 2 capsules = 500 mg). Batch NO.: 170507, Aida Pharmaceutical Company of Hangzhou. Contrast product: Azithromycin Batch NO.: 130352–200405 Content: 93.6%. National Institute for the Control of Pharmaceutical and Biological Products. Roxithromycin Batch NO.: 130557–200501 Content: 91.4%. National Institute for the Control of Pharmaceutical and Biological Products. Reagent: Methanol for HPLC. Fisher Company. Ammoum Acetate for AR. Shanghai Chemical Reagent CO., LTD. Instrument: Masslynx Waters 2695-Quattro micro API LC-MS-MS system.

4.2. Chromatographic conditions

HPLC Columns: Waters Xterra MS C_{18} (dp 5 μm, ID 2.1×100 mm). Column temperature: 25 °C. Mobile Phase: methanol- ammonium acetate (10 mM, 1% acetic acid), gradient elution: from 0 to 1.2 min the proportion of methanol was 47% (V), from 1.3 to 5.3 min the proportion of methanol was 83% (V), from 5.4 to 9.0 min the proportion of methanol was 47% (V), Flow rate: 0.3 ml/min.

4.3. MS spectrometry detection

Electrospray ionization mass spectroscopy (ESI-MS) was carried out on a Masslynx LC-MS-MS series system. The ESI ion source was set in positive ion polasity mode for acquiring all mass spectrometry data. The selective ion monitoring (SIM) was set at m/z 749.8 for azithromycin and m/z 837.9 for roxithromycin respectively. The fragmentor, drying gas flow, drying gas temperature, and capillary voltage were set to 40 V, 400 $L \cdot h^{-1}$, 300 °C, and 3000 V, respectively.

4.4. Sample preparation for HPLC injection

A 0.2 ml plasma sample was taken in a plastic stoppered tube, 50 μL of internal standard (roxithromycin 1.999 $\mu g \cdot mL^{-1}$) was added and shaken on a vortex mixer for 30 s, then extracted with 0.7 ml of methanol using a vortex for 3 min, and centrifuged at 14000 r for 10 min. The top organic layer 0.5 ml was transferred to another tube and ammonium acetate (30 mM) 70 μL was added and the mixture was then centrifuged at 14000 r for 5 min. The upper aliquot of this (10 μL) was injected into the LC-MS-MS.

4.5. Drug administration and sample collection

Twenty healthy male volunteers were enrolled in the study. The age was in a range of 20−26 years and the weight was in a range of 57−75 kg. On the basis of medical history, clinical examination and laboratory investigation (haematology, blood biochemistry and urine analysis), no subject had a history or evidence of hepatic, renal, gastrointestinal or haematologic deviations or any acute or chronic disease or drug allergy. The subject was instructed to abstain from taking any medication for at least 1 week prior to and during the study period. Informed consent was obtained from the subject after explaining the nature and purpose of the study.

Twenty healthy male volunteers were randomly divided into two groups. Using a crossover design, each person was given a single dose of 500 mg. There was a 1 week washout period between the two crossover periods. After an overnight fasting (10 h) subjects received a single dose (2×250 mg) of either formulation (reference or test) of azithromycin with 200 ml of water. A standard breakfast was taken 3 h after dosing. Water intake was allowed after 2 h of dose. Volunteers were ambulatory during the study but prohibited from strenuous activity. Approximately 3 ml blood samples for azithromycin assay were drawn into heparinized tubes through an indwelling cannula before (0 h) and 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0, 72.0, 108.0 and 144.0 h after dosing. Then plasma was separated and kept frozen at −85 °C until analysis.

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