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ORIGINAL RESEARCH

# Rapid and sensitive HPLC-MS/MS method for pharmacokinetic assessment of ribavirin in healthy Chinese

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ABSTRACT: A rapid and sensitive quantitative assay method was developed for determining ribavirin pharmacokinetic in human plasma. The chromatographic separation was achieved within 4.5 min using a SinoChrom ODS-BP column (4.6 × 150 mm, 5 μm) with acetonitrile–water (1 mmol/L ammonium acetate buffer, 0.1% formic acid; 15:85, v/v) at a constant flow rate of 0.8 mL/min. The MRM pairs were $m/z$ 245.2 → $m/z$ 113.1 for ribavirin and $m/z$ 226.1 → $m/z$ 152.1 for acyclovir (internal standard), respectively, with dwell times of 200 ms for each transition. The results showed calibration curve for ribavirin was linear over a concentration range of 1–1000 ng/mL. The lower limit of quantification (LLOQ) was 1 ng/mL ribavirin. Twenty healthy volunteers received a 300 mg oral dose of ribavirin. Blood samples were then collected up to 120 h postdosing. All plasma data were comodeled for ribavirin by using noncompartmental modeling. The single dose of ribavirin was well tolerated and no serious adverse effects occurred. The mean time to maximum concentration was about 1.25 h. The mean maximum concentration of drug in plasma for oral ribavirin was 250 ng/mL. The mean elimination half-life was 43.6 h. The present study describes a simple, specific, sensitive HPLC–MS/MS method for measuring plasma drug concentration and analyzing human pharmacokinetics of ribavirin. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: ribavirin; HPLC-MS/MS; quantitative assay; pharmacokinetics

## INTRODUCTION

Ribavirin, systematically named 1-(β-D-Ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide, is a synthetic purine nucleoside analog with a broad spectrum of antiviral activity. It is activated by cellular adenosine kinase to ribavirin 5′ mono-, di- and tri-phosphates, which have been demonstrated to either indirectly or directly inhibit viral replication by interfering with viral polymerase-mediated RNA synthesis (Vo *et al.*, 2003). A combination of ribavirin with either interferon α-2a/2b (Poynard *et al.*, 1998) or peginterferon α-2a/2b (Shiffman *et al.*, 2004) was widely accepted for the treatment of chronic hepatitis C. In addition, the use of ribavirin combined with other agents in patients triple-infected with human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis B virus (HBV) has been evaluated (Thomas, 2006). The antiviral effect of ribavirin has been observed to depend significantly on plasma concentration and dosage (Arase *et al.*, 2005; Lindahl *et al.*, 2005). Thus, the pharmacokinetic properties of ribavirin may hold tight links to the identification of improved therapeutic protocols (Dixit and Perelson, 2006). Following a single oral dose, the plasma concentration of ribavirin exhibits a three-phase profile—a rapid absorption phase, a rapid distribution phase, and a long terminal elimination phase (Glue, 1999). Today ribavirin is widely used for treatment of respiratory syncytial virus and HCV infections of humans in China. Unfortunately, no detailed pharmacokinetic data have been reported for this drug, although ribavirin has been recommended to be administered orally in doses of 150 or 300 mg three times a day (State Food and Drug Administration of China, 2006). In order to better understand the exposure of ribavirin in Chinese people, it is necessary to assess ribavirin pharmacokinetics and develop a rapid and sensitive method for the measurement of ribavirin in human plasma.

Many high-performance liquid chromatography (HPLC) based methods (HPLC-UV, LC–MS, etc.) have been

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**Abbreviations used:** HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus.

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reported for the determination of ribavirin in biological matrices (D'Avolio *et al.*, 2006; Granich *et al.*, 1989; Inoue *et al.*, 2004; Larrat *et al.*, 2003; Li *et al.*, 2007; Lin *et al.*, 2002; Liu *et al.*, 2006; Shou *et al.*, 2002; Svensson *et al.*, 2000; Yeh *et al.*, 2007; Yeh *et al.*, 2003; Yeh *et al.*, 2005). However, the reported methods (D'Avolio *et al.*, 2006; Inoue *et al.*, 2004; Li *et al.*, 2007; Lin *et al.*, 2002; Liu *et al.*, 2006; Shou *et al.*, 2002) were complicated and involved time-consuming sample preparation procedures, including drying and reconstitution. The HPLC analysis cycle time was greater than 12 min (Larrat *et al.*, 2003). These methods had an assay sensitivity of 10 ng/mL (Li *et al.*, 2007; Lin *et al.*, 2002; Shou *et al.*, 2002), 25 ng/mL (Svensson *et al.*, 2000), 78 ng/mL (Li *et al.*, 2007) or 100 ng/mL (Granich *et al.*, 1989; Larrat *et al.*, 2003; Yeh *et al.*, 2003) and thus had limited usefulness in the pharmacokinetic evaluation of ribavirin after oral administration at a dose of 300 mg. The present paper reports the development of a simple sensitive HPLC-MS/MS method, using a simplified pretreatment procedure without relying on evaporation, for determining ribavirin in human plasma. The assay described here required only a small sample volume, and it was validated and applied in the pharmacokinetic studies of ribavirin in healthy Chinese.

## MATERIALS AND METHODS

**Chemicals and instruments.** Methanol and acetonitrile of HPLC grade were purchased from Tedia (Fairfield, OH, USA). Ammonium acetate and formic acid of HPLC grade were purchased from Merck (Guangzhou, China). Ribavirin [1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide] (purity, 99%) and acyclovir (purity, 98%), which was used as an internal standard (IS), were purchased from Sigma-Aldrich (Branch, China). Ribavirin formulation (lot no. 060403; 100 mg/tablet) was obtained from Shichuan Medco Pharmaceutical Co. Ltd (Shichuan, China). Blank human plasma from healthy donors was obtained from the Blood Service Center of Guangzhou (Guangdong, China) or from healthy volunteers.

A Shimadzu liquid chromatograph model LC-20AB Prominence integrated system consisting of a multi-channel mobile phase degasser (DGU-20$A_3$), a column heater (CTO-20A), two pumps (LC-20AB pumps, Shimadzu) and a SinoChrom ODS-BP (4.6 × 150 mm, 5 μm particle size) column (Elite, Dalian, China) equipped with a guard column (Gemini $C_{18}$ 4.0 × 3.0 mm, Phenomenex®, USA) was used for chromatographic separation of ribavirin and internal standard. The autosampler was SIL-20AC from Shimadzu.

An API 4000 Q Trap mass spectrometer (AB/MDS-Sciex, USA) with a turboionspray (TIS) interface operating in positive ionization mode was used for the multiple reaction monitoring (MRM) LC–MS/MS analyses. Data were processed using the AB/MDS-Sciex Analyst 1.4.1 software (Foster City, CA, USA).

**HPLC and MS conditions.** The separation was carried out with an isocratic mobile phase of acetonitrile–water (1 mmol/L ammonium acetate buffer, 0.1% formic acid; 15:85, v/v) at a constant flow rate of 0.8 mL/min for 4.5 min. The column was maintained at 30°C. The autosampler temperature was set at 4°C. The mass spectrometric conditions were optimized for ribavirin and acyclovir by infusing a 200–500 ng/mL standard solution in 50% methanol (containing 0.1% formic acid and 0.001 M ammonium acetate) at 10 μL/min using a Harvard infusion pump (Harvard Apparatus, South Natick, MA, USA) directly connected to a 'Tee', where the compounds were mixed with a mobile phase mixture at 0.8 mL/min (85% mobile phase A and 15% mobile phase B) before entering the mass spectrometer.

The optimized MS conditions were as follow: TIS source temperature, 700°C; TIS voltage, 5000 V; curtain gas, 25; nebulizing gas (GS1), 55; TIS (GS2) gas, 60; collision energy (CE), 15 eV for ribavirin and 20 eV for acyclovir. The following precursor → product ion transitions were used for multiple reaction monitoring: ribavirin, $m/z$ 245.2 → 113.1 and acyclovir, $m/z$ 226.1 → 1→ 152.1, with a dwell time of 200 ms for each mass transition of the analyte and internal standard. The mass spectrometer was operated at unit mass resolution for both the first quadrupole and the third quadrupole.

**Standards and quality control (QC) samples.** Two primary stock solutions for ribavirin and acyclovir were each prepared in methanol (containing 20 mM ammonium acetate and 0.1% formic acid), respectively, at a concentration of 1.0 mg/mL in 20 mL glass vials. The stock solutions were kept refrigerated (4°C). The stock solution was serially diluted with 50% methanol (containing 20 mM ammonium acetate and 0.1% formic acid) to prepare standard working solutions at the desired concentrations. The calibration standards were freshly prepared by spiking an appropriate amount of the standard working solution into 0.1 mL human plasma. Seven non-zero calibration standards were prepared at concentrations of 1, 5, 25, 150, 300, 750 and 1000 ng/mL. QC samples were prepared at concentrations of 3.0, 200 and 750 ng/mL. An internal standard working solution containing 500 ng/mL of acyclovir was prepared from internal standard (IS) stock solutions with 50% methanol.

**Sample preparation.** Sample aliquots of 0.1 mL were transferred from the vials into corresponding 2 mL polypropylene vials. Internal standard working solution (10.0 μL) was then added to the samples. After the addition of 0.3 mL of acetonitrile, the mixture was vortex-mixed at high speed on an XW-80A Vortexer (Instrument Factory of Shanghai Medical University, China) for 1 min. The samples were centrifuged at approximately 10,000 rpm for 10 min. Approximately 0.1 mL of the supernatant was transferred into 2 mL glass vials, then 30 μL was injected to the HPLC column.

**Validation of the method.** The linearity was tested by assaying a set of seven calibrators (1, 5, 25, 150, 300, 750 and 1000 ng/mL) eight times and by calculating the measured concentration of each calibrator from the calibration curve. A plot of the percentage deviation from the calculated concentration against the nominal concentration was drawn and inspected for trends. The calibration curves (analyte peak area/IS peak area vs analyte concentration) were constructed using the least square linear regression fit ($y = a + bc$). The acceptance criterion was established to be >0.98 for the calibration curve coefficient of correlation ($r$).



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Precision and accuracy assays were carried out five times using three different concentrations (3.0, 200.0 and 750 ng/mL) on the same day and over five different days. The mean, standard deviation (SD) and accuracy of the intra-day and inter-day experiments were calculated. The accuracy is expressed as the relative error of measurement (RE, %):

$$\mathrm{RE}\ (\%) = \left( \frac{\text{Mean calculated concentration}}{\text{Nominal concentration}} \right) \times 100$$

The stability of the stock solutions and working solutions of ribavirin and acyclovir, which were stored at 4°C for 3 months and at room temperature (25°C) for 24 h, was tested by comparing the instrument response with that of freshly prepared solutions. The stability experiments of ribavirin in human plasma were carried out under four conditions: after three freeze–thaw cycles, after storage at room temperature for 24 h, at 4°C for 8 h in the autosampler, and at –70°C for 3 months. The stability of ribavirin in human plasma was investigated using five replicates of each of low, medium and high QC samples. Stability was determined by comparing the nominal concentration of ribavirin and the calculated concentration of test samples.

Specificity of the method was established by measuring six independent sources of control human plasma or plasma samples spiked with ribavirin and acyclovir (IS).

The extraction recoveries were determined at three concentration levels (3.0, 200 and 750 ng/mL) for ribavirin and at one concentration level for acyclovir (IS) by comparing the analyte peak areas obtained from the quality control samples ($n = 5$) after extraction with those obtained from the corresponding unextracted reference standards prepared at the same concentrations.

**Pharmacokinetic assessment.** The ethics permit was obtained from the Ethics Committee of Guangdong Provincial People's Hospital, Guangzhou, China. After explaining the aims and risks of the study according to the Helsinki–Tokyo Declaration, all participants signed a consent to this study. The pharmacokinetics of ribavirin tablet at a single oral dose of 300 mg was assessed. All subjects received a complete medical history, physical examination and routine laboratory tests, and all volunteers had no hepatic, renal, cardiac, hematologic or other diseases. There was no pregnancy or menstruation for female subjects. Smokers and alcoholics were excluded.

Blood samples were obtained over 120 h (at baseline and at 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4, 6, 8, 10, 12, 24, 36, 48, 72, 96 and 120 h post-dose) in test tubes containing heparin. Following centrifugation (3000***g*** × 10 min at 4°C), plasma was collected and stored immediately at –70°C until analysis. Ribavirin plasma concentration levels were determined using HPLC-MS/MS.

Pharmacokinetic calculations were based on plasma concentrations which exceeded the lower limit of quantification. The pharmacokinetic parameters were assessed by utilizing a nonlinear curve-fitting software package (DAS2.1, edited by Rui-yuan Sun, Wuhu, Anhui Province, China) using non-compartmental modeling. $C_{max}$ and $T_{max}$ were the observed values; the area under the concentration–time curve (AUC) was calculated according to the linear trapezoidal rule.

**Statistical methods.** Geometric mean and associated 95% confidence intervals (CIs) for AUC and $C_{max}$ (CIs were first determined using logarithms of individual values and then expressed as linear values), arithmetic mean and associated 95% CIs for apparent volume of distribution ($V_z$/F), apparent total body clearance ($CL_z$/F), and elimination half-life ($t_{1/2z}$), median value and associated range for $t_{max}$ were estimated for pharmacokinetic parameters. The coefficient of variation (CV) was calculated to express the variability of the pharmacokinetic parameters [(SD/mean) × 100]. The variances of pharmacokinetic parameters between male and female subjects were compared by analysis of variance (ANOVA). A $p$-value of ≥0.05 was considered significant. The variations of laboratory parameters to assess safety before and after administration were analyzed by paired $t$-test. Adverse effects were summarized with frequencies and percentages. Descriptive statistical analyses used SPSS version 11.5 for Windows (2004).

## RESULTS

### Ribavirin assay validation

The retention times of ribavirin and acyclovir (IS) were approximately 2.59 and 3.69 min, respectively. The representative LC-MS/MS chromatograms of human plasma spiked with IS acyclovir (Fig. 1) are shown; no interfering peak was observed in human plasma.

For acyclovir, the average extraction recovery was 90%, and the RSD was 5.1%. The average extraction recoveries of ribavirin were 88, 91 and 92% for quality control samples with low (3 ng/mL), medium (200 ng/mL) and high (750 ng/mL) concentrations examined, respectively. The RSD ranged from 3.9 to 5.2%.

The concentration range was 1–1000 ng/mL for ribavirin. The peak area ratio ($y$) of the analyte to IS was well correlated to the concentration ($C$). A representative calibration curve with linear regression (weighing of 1/concentration) for ribavirin is $y = 0.00398 + 0.07441C$ ($r = 0.9989$).

The analysis of independent low, medium and high quality control samples was used to determine intra-day and inter-day precision and accuracy of the assay. The intra-day RSD for ribavirin ranged from 2.4 to 4.3% and the accuracy from –8.7 to 8.4%. The inter-day RSD for ribavirin varied from 3.0 to 6.2% and the accuracy from –5.8 to 9.3%. The above results indicated that the method is reliable, reproducible and accurate.

Five quality control plasma samples were utilized to determine the sensitivity. The limit of quantitation (LLOQ) was 1 ng/mL for ribavirin. The stock solutions and working solutions of ribavirin and acyclovir (IS) stored at 4°C for 3 months and at room temperature (25°C) for 24 h showed good stability with RE ranging from –4.1 to 3.4%. The stability data of ribavirin in human plasma under four conditions are shown in



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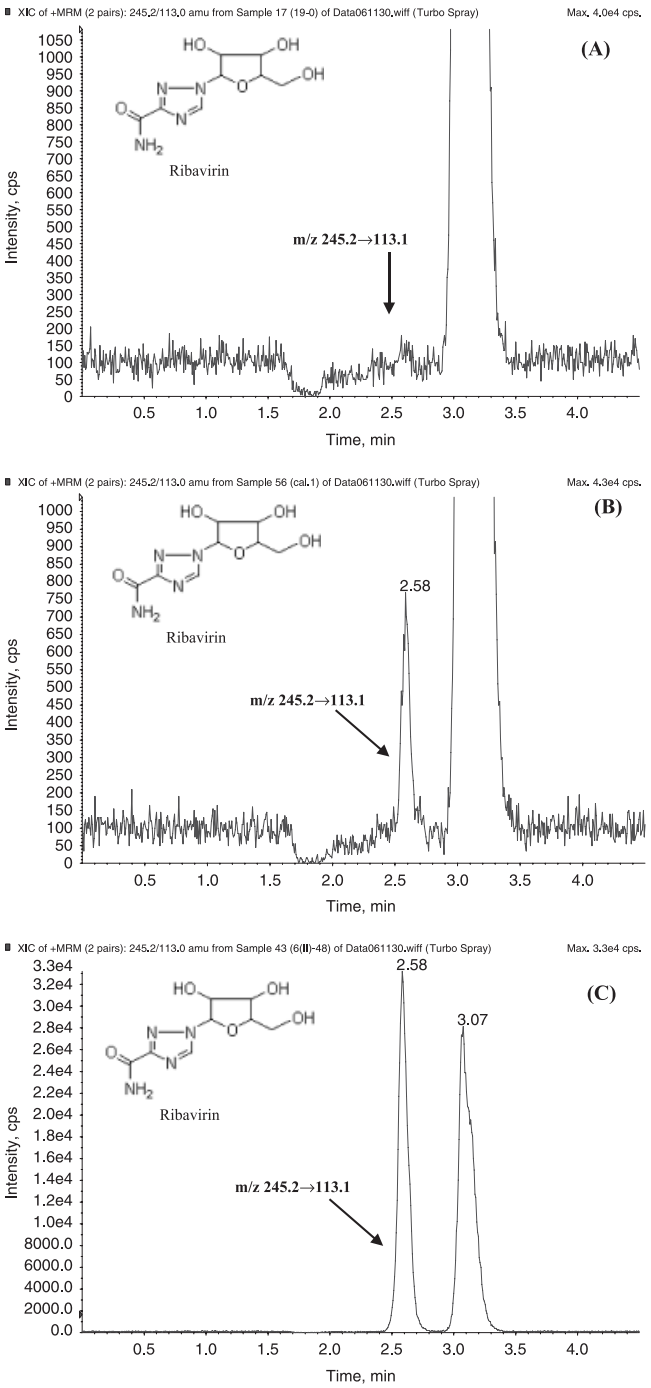


**Figure 1.** (A) Representative LC-MS/MS chromatograms of blank human plasma of ribavirin (*m/z* 245.2 → 113.1). (B) Representative LC-MS/MS chromatogram of LLOQ sample containing 1 ng/mL of ribavirin. (C) Representative LC-MS/MS chromatogram of a plasma sample at 48 h after dosing from a human volunteer containing 55.1 ng/mL of ribavirin.

Table 1. Five replicates of each of low, medium and high quality control samples were analyzed. As shown in Table 1, no significant degradation of ribavirin was observed under any of those conditions.

## Pharmacokinetics

A total of 20 healthy Chinese volunteers (male, 10; female, 10; age: mean ± SD, 25.1 ± 1.7 years; body mass index, 21.7 ± 1.5) were recruited in the study. There was no drop-out during the study. The main pharmacokinetic parameters of ribavirin are presented in Table 2; the mean plasma concentration–time curve is shown in Fig. 2.

Compared with in males, lower $V_z/F$, prolonged $t_{1/2z}$ and higher $C_{max}$ were observed in female subjects, which may be caused by body weight differences, but no statistical significance ($p > 0.05$) was found.

## DISCUSSION

We validated a new method to measure ribavirin plasma concentration. Compared with previously published methods (D'Avolio *et al.*, 2006; Inoue *et al.*, 2004; Li *et al.*, 2007; Lin *et al.*, 2002; Shou *et al.*, 2002) or another

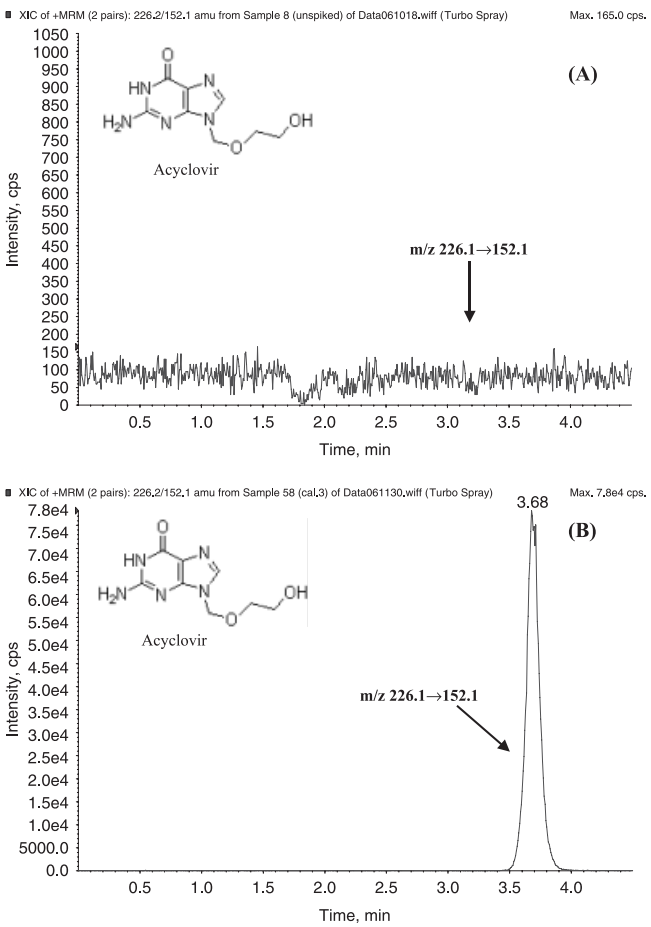


**Figure 2.** (A) Representative LC-MS/MS chromatograms of blank human plasma of acyclovir (*m/z* 226.1 → 152.1). (B) Representative LC-MS/MS chromatogram of a spiked plasma sample containing 45 ng/mL of acyclovir (IS).

**Table 1. Stability of ribavirin in human plasma**

| Experimental conditions | Parameter | QC concentrations (ng/mL) ($n$ = 5) | | |
|---|---|---|---|---|
| | | 3 | 200 | 750 |
| After three freeze–thaw cycles | Mean calculated concentration | 2.93 | 186 | 777 |
| | RSD (%) | 4.4 | 2.3 | 4.3 |
| | RE (%) | –2.1 | –7.3 | 3.6 |
| At room temperature for 24 h | Mean calculated concentration | 3.18 | 198 | 806 |
| | RSD (%) | 3.9 | 2.4 | 4.3 |
| | RE (%) | 6.1 | –1.4 | 7.5 |
| At 4°C for 8 h | Mean calculated concentration | 2.90 | 183 | 795 |
| | RSD (%) | 3.4 | 2.1 | 3.8 |
| | RE (%) | –3.2 | –8.9 | 8.7 |
| At –70°C for 3 months | Mean calculated concentration | 3.25 | 219 | 708 |
| | RSD (%) | 5.4 | 2.9 | 4.6 |
| | RE (%) | 8.1 | 9.8 | –6.9 |

**Table 2. Main pharmacokinetic parameters of ribavirin following a single oral dose of 300 mg ($n$ = 20)**

| Parameter | Mean or median | 95% CI or range | RSD (%) |
|---|---|---|---|
| $AUC_{0-120\,h}$ (ng h/mL) | 5797[a] | (4989, 6734) | 27.1 |
| $AUC_{0-\infty}$ (ng h/mL) | 6756[a] | (5793, 7881) | 26.7 |
| $C_{max}$ (ng/mL) | 250[a] | (205, 305) | 40.9 |
| $t_{1/2z}$ (h) | 43.6[b] | (36.6, 50.7) | 34.2 |
| $V_z$/F (L) | 2868[b] | (2300, 3437) | 41.8 |
| $CL_z$/F (L/h[1]) | 47.2[b] | (37.6, 56.8) | 43.6 |
| $T_{max}$ (h) | 1.25[c] | (1.0–2.5)[d] | — |

[a] Geometric mean.
[b] Arithmetic mean.
[c] Median.
[d] Range.

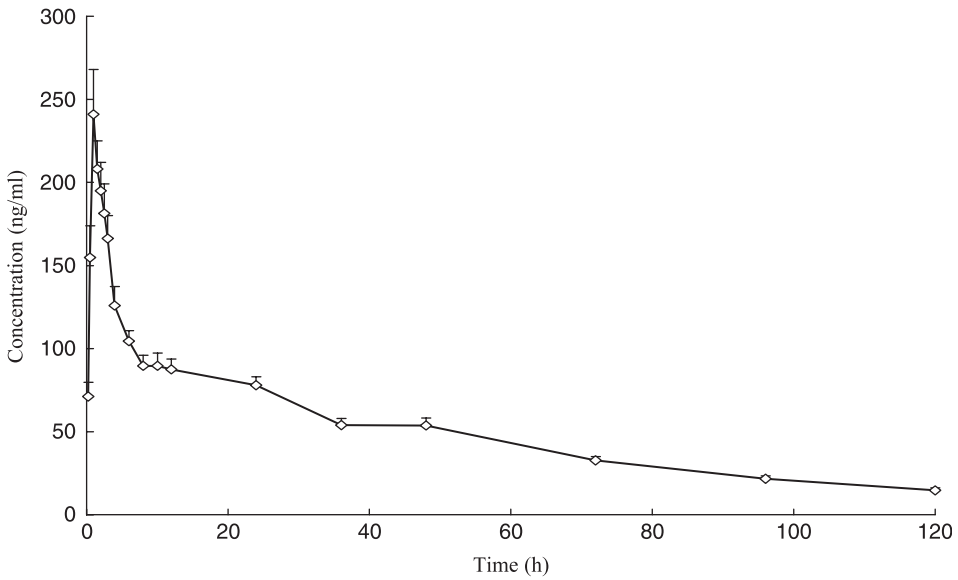


**Figure 3.** Mean plasma concentration–time profile of ribavirin following a single oral dose of 300 mg ($n$ = 20, arithmetic mean ± standard error).



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highly sensitive LC-MS/MS method (Liu *et al.*, 2006) with an LLOQ of 1 ng/mL, our assay provides a simplified sample pretreatment procedure. In these methods, plasma samples were cleaned with liquid–liquid phase or solid-phase extraction or protein precipitation, evaporated to dryness and reconstituted with HPLC mobile phase. These sample preparation methods are tedious. In contrast, the current LC–MS/MS method does not require liquid–liquid phase extraction, solid-phase extraction, drying or reconstitution. In addition, the current method is highly sensitive, requiring only 0.1 mL of plasma, with an LLOQ of 1 ng/mL. This sensitivity is sufficient to measure plasma concentrations of ribavirin for at least eight half-lives after the $C_{max}$, following oral administrations at 300 mg in Chinese healthy volunteers (250 μg/L). Another advantage related to the validation of our method was the short analysis time run. In fact, the ribavirin peak eluted in 2.6 min and the system was ready for a new analysis after 5 min, including wash-out and conditioning steps. Moreover, the calibration curve of our method covered a wide range of ribavirin concentrations from 1 to 1000 ng/mL.

Following a single oral 300 mg dose, the plasma concentration of ribavirin exhibited a similar profile to that described previously (Glue, 1999)—a rapid absorption phase, followed by a rapid distribution phase and a long terminal elimination phase. Mean $t_{max}$ value was about 1.25 h, indicating rapid absorption of ribavirin, and it was generally similar to other reported data (Glue *et al.*, 2000; Khakoo *et al.*, 1998). These studies indicate that ribavirin has a large volume of distribution (Mean: 2868 L) with a long mean elimination half-life of 43.6 h. These findings may indicate that ribavirin pharmacokinetics is not necessarily affected by race. Ribavirin is eliminated by metabolism as well as renal elimination, the latter accounting for about 5–15% of single dose elimination (Glue *et al.*, 2000). Metabolism thus plays a key role in ribavirin elimination (Dixit and Perelson, 2006). Although the metabolism enzyme CYPs has high intersubject variability in intrinsic metabolic capacity across the population, the gastrointestinal tract, but not the liver, appears to be the major site of first-pass elimination of ribavirin (Yeh *et al.*, 2003). Therefore, the parameters that describe single-dose pharmacokinetics may not be applicable to a multiple-dose case (Dixit and Perelson, 2006). A multiple-dose pharmacokinetic evaluation might be needed to determine the optimal dose in Chinese population.

In conclusion, an LC–MS/MS assay for the measurement of ribavirin in human plasma has been established. The method is specific, highly sensitive with an LLOQ of 1 ng/mL, accurate over a concentration range of 1–1000 ng/mL with a simple sample preparation. The method has been used to quantify levels of ribavirin in human plasma for pharmacokinetic assessment. Following a single dose of 300 mg, the plasma pharmacokinetics of ribavirin in healthy Chinese volunteers was similar to previous reports in western populations.

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