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Journal of Chromatography B, 832 (2006) 17–23
JOURNAL OF CHROMATOGRAPHY B
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Sensitive and specific LC–MS/MS method for the simultaneous measurements of viramidine and ribavirin in human plasma

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Received 11 January 2005; accepted 3 December 2005

Abstract

Viramidine is a prodrug of ribavirin. To facilitate pharmacokinetics studies of viramidine in man, a sensitive and specific LC–MS/MS method for the simultaneous analyses of viramidine and ribavirin in human plasma was developed and validated. The method involved the addition of [^{13}C]viramidine and [^{13}C]ribavirin as internal standards, protein precipitation with acetonitrile, HPLC separation, and quantification by MS/MS system using positive electrospray ionization in the multiple reaction monitoring mode (MRM). The precursor → product ion transitions were monitored at 245 → 113, 250 → 113, 244 → 112, and 249 → 112 for ribavirin, [^{13}C]ribavirin, viramidine, and [^{13}C]viramidine, respectively. The calibration curves for viramidine and ribavirin were linear over a concentration range of 1–1000 ng/mL. For both viramidine and ribavirin, the lower limit of quantification (LLOQ) was 1 ng/mL. For viramidine, intra- and inter-day analyses of QC samples at 1, 5, 250, and 1000 ng/mL indicated good precision (%CV between 1.0 and 7.0%) and accuracy (%bias between −4.3 and 5.2%). For ribavirin, intra- and inter-day analyses of QC samples at 1, 5, 250, and 1000 ng/mL indicated similar precision (%CV between 0.8 and 8.3%) and accuracy (%bias between −5.8 and 9.4%). Both viramidine and ribavirin were stable in human plasma stored at room temperature for at least 3 h, 4 °C for at least 6 h, and for at least three freeze–thaw cycles. This accurate and highly specific assay provides a useful method for evaluating the pharmacokinetics of viramidine and ribavirin in man following administration of viramidine.

Keywords: Viramidine; Ribavirin; Specificity; Sensitivity; Hepatitis C; Measurement

1. Introduction

Ribavirin is a purine nucleoside analog (Fig. 1) with broad-spectrum activity against a variety of DNA and RNA viral infections [1–3]. In combination with either interferon alpha (IFN-α) or pegylated IFN-α, the clinical efficacy of ribavirin in the treatment of chronic hepatitis C virus (HCV) infection, in terms of sustained virologic response, has been shown to be approximately 41–47% [4,5] and 54–56% [6,7], respectively.

A dose-limiting adverse effect associated with the long-term therapeutic use of ribavirin is the potential for causing hemolytic anemia. A significant portion of ribavirin in the circulation is transported into erythrocytes [8] and metabolized into various phosphorylated derivatives [9]. Due to the lack of phosphatase activity in erythrocytes, these phosphorylated metabolites of ribavirin become trapped intracellularly, accumulate over time, and eventually result in hemolytic anemia [10,11]. This adverse effect often necessitates reduction of dose and/or discontinuation of ribavirin therapy in a significant proportion of patients. A second generation analog of ribavirin, therefore, which retains clinical efficacy but demonstrates a lower potential for causing hemolytic anemia would be highly desirable.

Viramidine is an analog and prodrug of ribavirin, which in the primate animal model, has been shown to be retained in the liver and converted to ribavirin. Cynomolgus monkeys given daily oral doses of [^{14}C]viramidine or [^{14}C]ribavirin for 10 days were observed to have higher hepatic but lower RBC and plasma drug levels after [^{14}C]viramidine than [^{14}C]ribavirin dosing [1]. Since the liver is the target for hepatitis C infection and RBCs are the site of ribavirin toxicity, viramidine could potentially provide the same therapeutic efficacy as ribavirin but with lower risk of hemolytic anemia.

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doi:10.1016/j.jchromb.2005.12.034

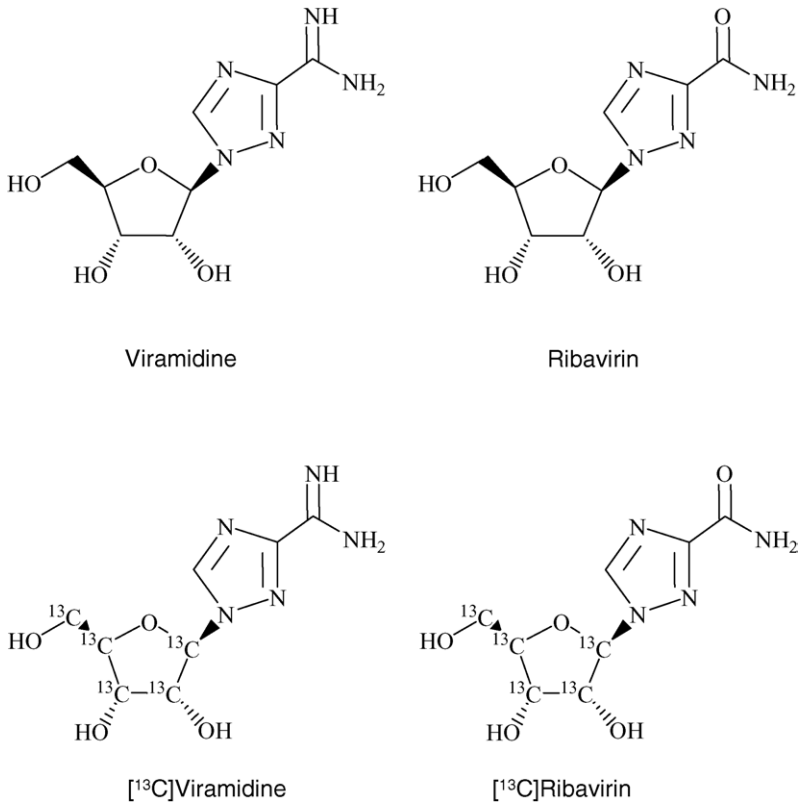

Fig. 1. Chemical structures of viramidine and ribavirin.

Accurate derivation of PK parameters for ribavirin is limited by the lack of a sensitive assay with high specificity. Austin et al. reported a radioimmunoassay for the measurement of ribavirin that is capable of determining plasma concentration as low as 0.01 μM (2.45 ng/mL) [12]. However, the assay lacks specificity and cross-reacts with many ribavirin metabolites, thus, limiting its usefulness in pharmacokinetic studies. Another method for quantitating ribavirin by HPLC with UV detection was reported by Paroni et al. [13]. This method, however, has an assay sensitivity of 100 ng/mL, which is inadequate for the pharmacokinetic evaluation of ribavirin in animals and man following oral administration. We previously reported an LC–MS/MS ribavirin assay sensitive to 10 ng/mL of ribavirin in rat and monkey plasmas, which has been used to evaluate pharmacokinetics of ribavirin in animals [14]. Recently, an LC–MS/MS method using a silica column, with an LLOQ at 10 ng/mL for ribavirin analysis in human plasma and serum, was reported by Naidong and co-workers [15]. For simultaneous analyses of ribavirin and viramidine, we recently reported an LC–MS/MS method for the simultaneous determinations of ribavirin and viramidine in rat and monkey plasmas [16], as well as human serum [17], with an LLOQ of 10 ng/mL.

In this report, a more sensitive LC–MS/MS method for the simultaneous determination of viramidine and ribavirin in human plasma, with an LLOQ of 1 ng/mL, is described. This assay has been validated and successfully used to evaluate the pharmacokinetic dispositions of viramidine and ribavirin in man following oral administration of viramidine.

2. Method

2.1. Chemicals

Viramidine (99.7%), [^{13}C]viramidine (97.8%), ribavirin (99.8%), and [^{13}C]ribavirin (90.8%) (Fig. 1) were supplied by Valeant Research & Development, Costa Mesa, CA. Acetonitrile (HLPC grade), methanol (HPLC grade), and water (HPLC grade) were purchased from EM Science. Formic acid was purchased from JT Baker.

2.1.1. Drug administration and plasma sample collection

In a single-dose pharmacokinetic study, six healthy volunteers each received an oral 600 mg dose of viramidine. Venous blood samples (10 mL aliquots) were collected into heparinized tubes via an indwelling catheter pre-dose and at the following times: 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, 24, 36, 48, 60, 72, 96, 120, 144, 168, 216, 264, 336, 504, 576, and 672 h post-dose.

2.2. Sample preparation

Duplicate calibration standards were prepared at seven concentration levels, ranging from 1 to 1000 ng/mL, by spiking ribavirin and viramidine standard spiking solutions into blank human plasma (Bioreclamation Inc., Hicksville, NY). Four levels of QC samples were prepared by spiking the QC solution into human plasma blanks (5%, v/v) at 1, 5, 25, and 1000 ng/mL.

QC samples and plasma were stored at −70 °C until analysis.

The internal standard (10 μL, 2 μg/mL each of [^{13}C]viramidine and [^{13}C]ribavirin in water), water (10 μL), and acetonitrile (400 μL) were added to 100 μL aliquot of human plasma. The mixture was vortexed for 1 min, and then centrifuged at 13,000 rpm for 10 min. The supernatant (200 μL) was transferred to a 96-well plate and evaporated to dryness at 25 °C. The residue was reconstituted in 200 μL of water (HPLC grade), and a 20 μL aliquot of the mixture was injected into the LC–MS/MS system.

3. LC–MS/MS system

The LC–MS/MS system consisted of a high-throughput liquid chromatographic (HTLC) system (Cohesive Technologies, Franklin, MA) and an API 4000 mass spectrometer (Applied Biosystems, Foster City, CA). The LC separation was accomplished using a Waters Polarity^{TM} column, C18, 4.6 mm × 150 mm, 5 μm (Waters Associates, Watersbury, MA), maintained at room temperature. Mobile phase A consisted of 0.1% formic acid in water. Mobile phase B consisted of 0.1% formic acid in methanol. The gradient conditions were 100% A between 0 and 1 min, 95% A and 5% B between 1 and 3.5 min, returned to 50% A and 50% B between 3.5 and 5 min, maintained at 50% A and 50% B between 5 and 6.5 min, ramped to 100% A between 6.5 and 6.6 min and maintained at 100% A between 6.6 and 8 min. The mobile phases were delivered at 0.9 mL/min.

The precursor → product ion transitions were monitored at 245 → 113, 250 → 113, 244 → 112, and 249 → 112 for ribavirin, [^{13}C]ribavirin, viramidine, and [^{13}C]viramidine, respectively. Each transition was alternately monitored with a dwell time of 150 ms. Collision-activated dissociation (CAD) gas was set at 6 psi and curtain gas was set at 15 (arbitrary unit). The TurboIon Spray (TIS) source temperature (TEM) was maintained at 650 °C, and the IonSpray voltage (IS) was set at 5000 V. The nebulizer (GS1) and TIS (GS2) were set at 50 and 60 psi, respectively. Unit resolution was applied for both Q1 and Q3.

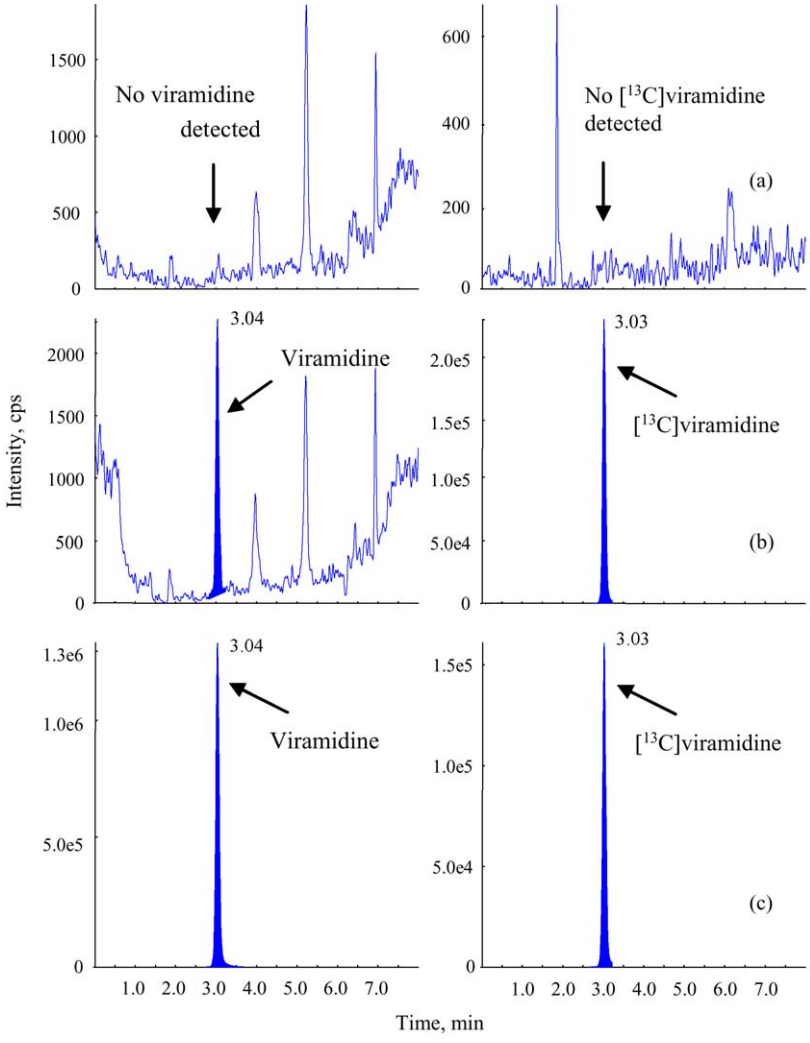

Fig. 2. Typical chromatogram of viramidine in extracted human: (a) plasma blank; (b) 1 ng/mL standard; and (c) 100 ng/mL standard.

3.1. Method evaluation

Precision (%CV) and accuracy (%bias) were calculated from the back-calculated concentrations of five standard curves prepared from plasma. The LLOQ was the lowest concentration in the standard curve, where the %CV values (10 replicates) and bias from the back-calculated concentrations were <20%. The specificity of the assay was established by the lack of interference at the retention times of viramidine, ribavirin, and their internal standards. The recoveries of ribavirin and viramidine were determined by comparing the peak area ratio of extracted standards with standards spiked after extraction, at the same nominal concentrations (low, medium, and high).

Freeze-and-thaw stability was determined by freezing and thawing the low- and high-QC samples for three cycles before analysis, with at least a 12 h interval between each cycle. Short-term stability was measured by leaving QC samples on bench for a certain time period before extraction. Post-injection stability of ribavirin and viramidine were determined for 24 h at room temperature.

3.2. Pharmacokinetic analysis

Plasma concentrations equal to or above LLOQ were used for pharmacokinetic analysis using a model-independent method (WinNonlin 4.0). The maximum plasma concentration (C_{max}) and time of C_{max} (T_{max}) were the observed values. The area under the plasma concentration-versus-time curve, from time zero to the time of the final measurable sample, [AUC(tf)] was calculated using the linear trapezoidal method. The elimination rate constant (K) was estimated as the slope of the regression of the log concentration-versus-time curve. Half-life ($t_{1/2}$) was calculated by dividing 0.693 by K.

4. Results

Typical LC–MS/MS chromatograms for viramidine and ribavirin compared with internal standards extracted from human plasma are shown in Figs. 2 and 3, respectively. The standard curve was obtained by fitting the ratio of peak height of viramidine or ribavirin to that of the internal standard against the

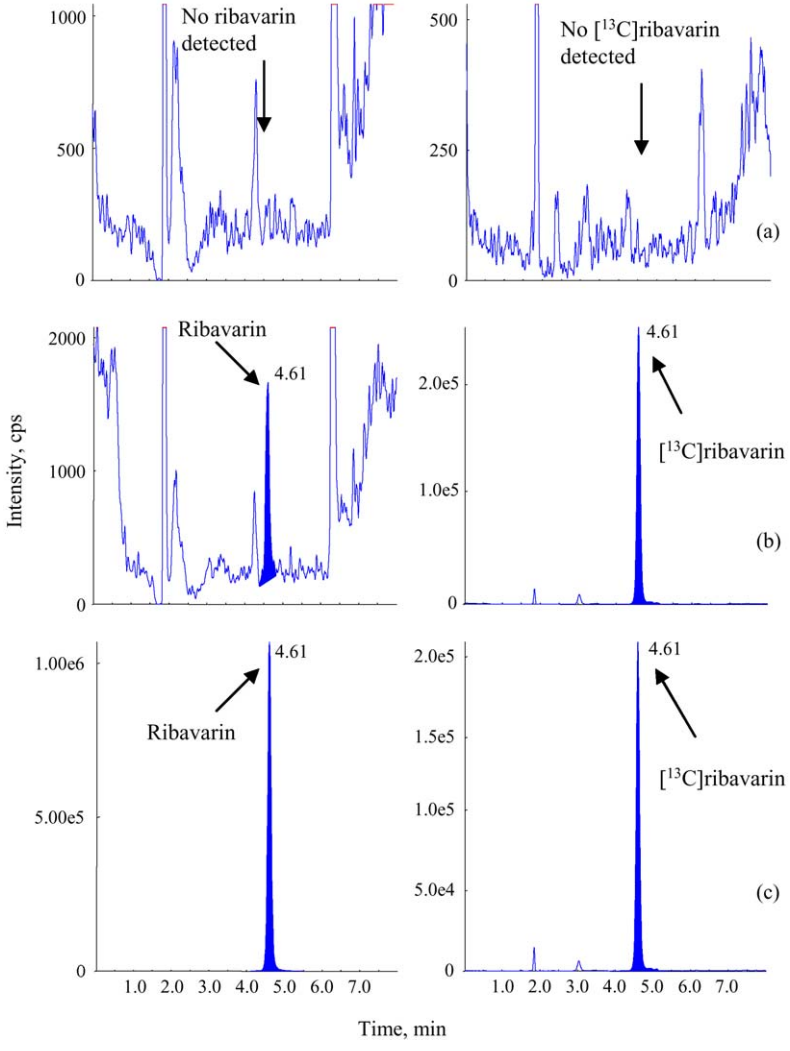

Fig. 3. Typical chromatogram of ribavirin in extracted human: (a) plasma blank; (b) 1 ng/mL standard; and (c) 1000 ng/mL standard.

Table 1
Linearity for viramidine and ribavirin

Analyte	Assay no.	Regression	Slope	Intercept	Correlation coefficient
Viramidine	09AE	$1/X^2$	0.00921	0.00124	0.9980
	10AE		0.00930	0.00602	0.9983
	11AE		0.00915	0.00113	0.9984
	12AE		0.00934	0.00134	0.9987
	13AE		0.00936	0.00145	0.9988
Ribavirin	09AE	$1/X^2$	0.00589	0.00152	0.9989
	10AE		0.00584	0.00731	0.9983
	11AE		0.00599	0.00710	0.9979
	12AE		0.00597	0.00362	0.9984
	13AE		0.00587	0.00100	0.9986

concentration (1–1000 ng/mL) of viramidine or ribavirin added, using the $1/X^2$ linear regression plots.

4.1. Viramidine

The correlation coefficients were ≥0.9980 in human plasma (Table 1). There were no interfering peaks in blank plasma at the retention time of either viramidine or the internal standard, indicating that the LC–MS/MS analysis for viramidine was highly selective. There was no matrix effect when peak ratio of viramidine/[^{13}C]viramidine was evaluated following extraction using six different lots of control plasma (mean precision <1.8%). Calibration standards showed good precision (%CV between 1.1 and 4.1%) and accuracy (%bias between −5.8 and 9.0%) from 1 to 1000 ng/mL (Table 2). Intra- and inter-day precision and accuracy of the method were validated at 1, 5, 250, and 1000 ng/mL. The results for inter-assay (Table 3) and intra-assay (Table 4) demonstrated good precision (%CV between 1.0 and 7.0%) and accuracy (%bias between −4.3 and 5.2%). Mean recovery of viramidine (5, 250, and 1000 ng/mL) from human plasma was 74.2%. Viramidine in human plasma was found to be stable at room temperature for at least 3 h (<11% change), at 4 °C for at least 6 h (<2% change), and for at least three freeze–thaw cycles (<6% change).

4.2. Ribavirin

The correlation coefficients were ≥0.9979 in human plasma (Table 1). There were no interfering peaks in blank plasma at the retention time of either ribavirin or the internal standard, indicating that the LC–MS/MS analysis for ribavirin was selective. There was no matrix effect when peak ratio of ribavirin/[^{13}C]ribavirin was evaluated following extraction with six different lots of control plasma (mean precision <1.4%). Calibration standards showed good precision (%CV between 1.4 and 5.5%) and accuracy (%bias between −6.1 and 5.9%) from 1 to 1000 ng/mL (Table 2). Intra- and inter-day precision and accuracy of the method were validated at 1, 5, 250, and 1000 ng/mL. The results for inter-assay (Table 3) and intra-assay (Table 4) demonstrated good precision (%CV between 0.8 and 8.3%) and accuracy (%bias between 5.8 and 9.4%). Mean recovery of ribavirin (5, 250, and 1000 ng/mL) from human plasma was determined to be 96.8%. Ribavirin in human plasma was found to be stable at room temperature for at least 6 h (<12% change) and for at least three freeze–thaw cycles (<12% change).

Table 2
Standard curve accuracy and precision for viramidine and ribavirin

Analyte	Parameter	Nominal concentration (ng/mL)						
		1.00	5.00	25.0	100	250	500	1000
Viramidine	*N*	10	10	10	10	10	10	10
	Mean	1.00	5.05	24.5	109	243	504	942
	%CV	4.11	3.90	1.09	1.73	1.55	2.02	2.21
	%Bias	0.00	1.00	−2.00	9.00	−2.80	0.80	−5.80
Ribavirin	*N*	10	10	10	10	10	10	10
	Mean	0.988	5.27	25.0	106	243	492	939
	%CV	5.50	3.55	2.84	1.37	1.67	2.83	3.25
	%Bias	−1.20	5.40	0.12	5.90	−2.60	−1.54	−6.06

Table 3
Inter-assay accuracy and precision for QC samples

Analyte	Parameter	LLOQ-QC (1 ng/mL)	Low-QC (5 ng/mL)	Mid-QC (250 ng/mL)	High-QC (1000 ng/mL)
Ribavirin	*N*	18	18	18	18
	Mean	1.02	5.46	262	943
	%CV	6.95	3.63	1.75	2.01
	%Bias	2.00	9.20	4.80	−5.70
Viramidine	*N*	18	18	18	18
	Mean	0.973	5.00	263	957
	%CV	6.42	4.20	3.95	2.92
	%Bia	−2.7	0.0	5.2	−4.3

Table 4
Intra-assay accuracy and precision for QC samples

Analyte	Parameter	LLOQ-QC (1 ng/mL)	Low-QC (5 ng/mL)	Mid-QC (250 ng/mL)	High-QC (1000 ng/mL)
Ribavirin	*N*	6	6	6	6
	Mean	1.05	5.47	262	942
	%CV	8.3	2.3	1.3	0.8
	%Bias	5.0	9.4	4.8	−5.8
Viramidine	*N*	6	6	6	6
	Mean	0.977	4.95	257	958
	%CV	7.0	3.2	2.3	1.0
	%Bias	−2.3	−1.0	2.8	−4.2

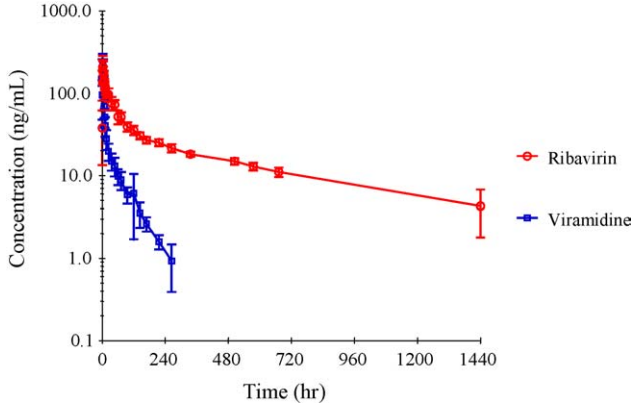

Fig. 4. Plasma concentration–time profiles for viramidine and ribavirin in man following oral administration of viramidine (600 mg).

4.3. Pharmacokinetic studies

This method was applied to the analysis of human plasma from healthy subjects after a single oral dose of 600 mg of viramidine. The mean plasma concentration–time curves for viramidine are shown in Fig. 4. Due to the excellent sensitivity of the assay, with an LLOQ of 1 ng/mL for both viramidine and ribavirin, serum concentrations were determined up to 248 h for viramidine and 1184 h for ribavirin. T_{max} was within 1.5–3.0 h for both viramidine and ribavirin. Results indicate that oral viramidine was absorbed and rapidly converted to ribavirin. Ribavirin AUC (22.5 μg h/mL) was 7.44 times the viramidine AUC (3.05 μg h/mL), indicating extensive conversion of viramidine to ribavirin in man.

5. Discussion

Until recently, most analytical methods for the determination of drugs in biological fluids were based on GC or HPLC methods. The introduction of an HPLC–API/MS/MS system (HPLC combined with atmospheric pressure ionization tandem mass spectrometry) has provided new techniques for developing a more sensitive and specific assaying method. Previously, we reported an LC–MS/MS method for the determination of ribavirin in rat and monkey plasmas using a silica column for HPLC separation [14]. In this method, plasma samples were cleaned with liquid–liquid phase extraction, evaporated to dryness, reconstituted with HPLC mobile phase, and HPLC analyzed. This sample preparation method is tedious and requires 0.2 mL of plasma. In contrast, the current LC–MS/MS method does not require liquid–liquid phase extraction, and only requires 0.1 mL of plasma. Furthermore, the LC–MS/MS method is highly specific, with an LLOQ of 1 ng/mL. This sensitivity is sufficient to measure plasma concentrations of ribavirin for at least nine half-lives after the C_{max}, following intravenous administrations at 30 mg/kg in rats (20.5 μg/mL) and monkeys (12.5 μg/mL).

Recently we have reported an LC–MS/MS method for the simultaneous determinations of ribavirin and viramidine in rat and monkey plasmas [16], as well as in human plasma [17]. The sensitivity of the method was similar to that reported previously for ribavirin, with an LLOQ of 10 ng/mL for both ribavirin and viramidine. However, only one plasma sample (0.05 mL) preparation was required for the analysis of both analytes, thus using less plasma sample overall and, more importantly, significantly reducing the time required for sample preparation and MS analysis.

In the present study, we describe the development and validation of a much more sensitive LC–MS/MS for the simultaneous determinations of viramidine and ribavirin concentrations with an LLOQ of only 1 ng/mL. This higher sensitivity was achieved by: (1) utilization of an API 4000 mass spectrometer instead of API 3000; (2) removal of perchloric acid during the protein precipitation step of the previous method; and (3) the switch from normal-phase chromatography to reverse-phase chromatography for better separation of ribavirin and viramidine from the endogenous peaks (most likely uridine and cytidine). With this sensitive LC–MS/MS method, we have been able to determine plasma concentrations up to 248 h for viramidine and 1184 h for ribavirin, as compared to 72 h for viramidine and 168 h for ribavirin, previously.

6. Conclusions

A very sensitive LC–MS/MS assay for simultaneous measurements of viramidine and ribavirin in human plasma was established, validated and shown to be specific, precise, and accurate over a concentration range of 1–1000 ng/mL. Viramidine and ribavirin were stable at 4 °C for at least 6 h and after three freeze–thaw cycles. The method was found to be useful for the simultaneous determinations of plasma concentrations of viramidine and ribavirin in man following oral administration of viramidine.

References

[1] C. Lin, L. Yeh, D. Vitarella, Z. Hong, Antiviral Chem. Chemother. 14 (2003) 145.
[2] J.T. Witkowski, R.K. Robins, R.W. Sidwell, L.N. Simon, J. Med. Chem. 15 (1972) 1150.
[3] R.W. Sidwell, J.H. Huffman, G.P. Khare, L.B. Allen, J.T. Witkowski, R.K. Robins, Science 177 (1972) 705.
[4] J.G. McHutchison, S.C. Gordon, E.R. Schiff, M.L. Shiffmann, W.M. Lee, V.K. Rustgi, Z.D. Goodman, M.H. Ling, S. Cort, J.K. Albrecht, N. Eng. J. Med. 339 (1998) 1485.
[5] T. Poynard, P. Marcellin, S.S. Lee, C. Niederau, G.S. Minuk, G. Ideo, V.H. Bain, J. Heathcote, S. Zeuzem, C. Trepo, J. Albrecht, Lancet 352 (1998) 1426.
[6] M.W. Fried, M.L. Shiffmann, K.R. Reddy, C. Smith, G. Marinos, F.L. Gonçales Jr., D. Haussinger, M. Diago, G. Carosi, D. Dhumeaux, A. Craxi, A. Lin, J. Hoffaman, J. Yu, N. Eng. J. Med. 347 (2002) 975.
[7] K.L. Lindsay, C. Trepo, T. Heintges, M.L. Shiffmann, S.C. Gordon, J.C. Hoefs, E.R. Schiff, Z.D. Goodman, M. Laughlin, R. Yao, J.K. Albrecht, Hepatology 34 (2001) 395.
[8] O.L. Laskin, J.A. Longstreth, C.C. Hart, D. Scavuzzo, C.M. Kalman, J.D. Connor, R.B. Roberts, Clin. Pharmacol. Ther. 41 (1987) 546.
[9] T. Page, J.D. Connor, Int. J. Biochem. 22 (1990) 379.
[10] E. Connor, S. Morrison, J. Lane, J. Oleske, R.L. Sonke, J. Connor, Antimicrob. Agents Chemother. 37 (1993) 532.
[11] J.J. Lertora, A.B. Rege, J.T. Lacour, N. Ferencz, W.J. George, R.B. VanDyke, K.C. Agrawal, N.E. Hyslop Jr., Clin. Pharmacol. Ther. 50 (1991) 442.
[12] R.K. Austin, P.E. Trefts, M. Hintz, J.D. Connor, M.F. Kagnoff, Antimicrob. Agents Chemother. 24 (5) (1983) 696.
[13] R. Paroni, M. Del Puppo, C. Borghi, C.R. Sirtori, M. Galli Kienle, Int. J. Clin. Pharmacol. Ther. Toxicol. 27 (1989) 302.
[14] C. Lin, L. Yeh, J. Lau, J. Chromatogr. B. 779 (2002) 241.
[15] W.Z. Shou, H.Z. Bu, T. Addison, X. Jiang, W. Naidong, J. Pharm. Biomed. Anal. 29 (2002) 83.
[16] C. Lin, K. Luu, D. Laurenco, L. Yeh, Antimicrob. Agents Chemother. 47 (2003) 2458.
[17] C. Lin, L. Philips, C. Xu, L. Yeh, J. Clin. Pharmacol. 44 (2004) 265.