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Specific, sensitive and accurate LC–MS/MS method for the measurement of levovirin in rat and monkey plasma

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Abstract

Levovirin is a guanosine nucleoside analogue and the L-enantiomer of ribavirin. Levovirin has a better safety profile than ribavirin, exerts similar immunomodulatory effects in a mouse efficacy model, and may provide a better therapeutic option than ribavirin in patients with chronic hepatitis C virus (HCV) infection. To facilitate pharmacokinetic studies, a LC–MS/MS method for the analysis of levovirin in rat and monkey plasma was developed and validated. The method involved adding ICN 10537 as an internal standard, protein precipitation with acetonitrile followed by separation on an Intersil Silica column, and quantification by a MS/MS system equipped with positive electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode. The MS/MS reaction was selected to monitor the $245 \rightarrow 113$ and $259 \rightarrow 128$ transitions for levovirin and internal standard, respectively. The calibration curve was linear over a concentration range of 10–5000 ng/ml. The limit of quantitation was 10 ng/ml, the coefficient of variation (CV) was 3–5%, and the bias was 3–6%. Intra- and inter-day analysis of QC samples at 30, 1500 and 3500 ng/ml indicated that the method was precise (CV $< 6\%$) and accurate (bias $< 9\%$). Levovirin in rat and monkey plasma was stable at 5 °C for at least 24 h, 0 °C for at least 4 h, and after three freeze–thaw cycles. This specific, accurate and precise assay is useful in the study the pharmacokinetic characteristics of this compound. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Levovirin; Specificity; Sensitivity; Hepatitis C; Measurement

1. Introduction

Levovirin (1-β-L-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) is a guanosine nucleoside analog and the L-enantiomer (molecular mirror image) of ribavirin. Levovirin was discovered in the course of identifying compounds which retain those properties deemed critical in the treatment of chronic hepatitis C virus (HCV) infections, but which do not share the toxicity profile of ribavirin [1].

Ribavirin (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) is a purine nucleoside analog with a broad spectrum of efficacy against a variety of DNA and RNA virus infections [2,3]. It has demonstrated clinical efficacy as monotherapy in the treatment of respiratory syncytial virus (RSV) infection and as combination therapy with inter-

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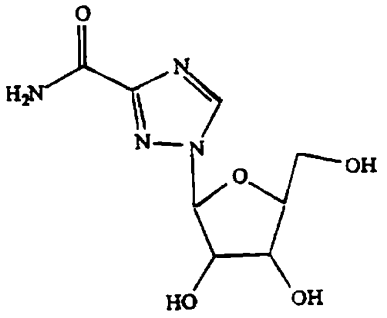
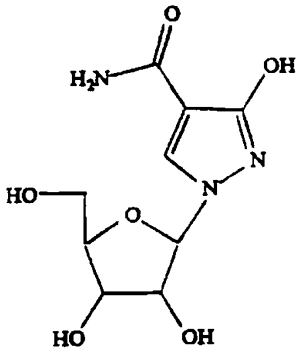
feron alpha (IFN-α) in the treatment of chronic HCV infection. It is widely accepted that ribavirin is a pleiotropic antiviral agent having multiple biological activities which may individually or collectively contribute to its clinical utility in treating viral infections. Numerous studies have suggested that the in vivo activity of ribavirin may be ascribed to at least two distinct activities: direct versus indirect antiviral effects [4]. The direct antiviral activity of ribavirin is consistent with its intracellular metabolic profile in that ribavirin converted to mono-, di- and tri-phosphorylated metabolites, characteristic of all known nucleoside inhibitors of viral infections [5]. Ribavirin may also elicit indirect antiviral effects by promoting T-cell mediated immunity [6–8] or by affecting intracellular GTP concentration by inhibiting host enzyme inosine monophosphate dehydrogenase (IMPDH) [9].



LEVOVIRIN



ICN 10537 (INTERNAL STANDARD)

Fig. 1. Molecular structures of levovirin and internal standard (ICN 10537).

It has been demonstrated that levovirin can stimulate immune responses (enhanced antiviral Th1 cytokine expression) against viral infections without any detectable direct antiviral effect [10]. Levovirin also suppresses Th2 cytokines, which are associated with the persistence of chronic HCV infection. Like ribavirin, levovirin also lowers the serum ALT level in a mouse hepatitis model [11]. In recent toxicology studies, levovirin did not appear to result in adverse effects while ribavirin caused anemia at the same dose level [12]. In contrast to ribavirin, levovirin did not accumulate in erythrocytes and did not lead to anemia [12]. Since levovirin has a better safety profile than ribavirin and exerts similar immunomodulatory effects in a mouse efficacy model, it may provide a better therapeutic option for patients with chronic HCV infection.

This report describes a LC–MS/MS method for the determination of levovirin in rat and Cynomolgus monkey plasma. This assay was validated and used in pharmacokinetic studies in rats and monkeys.

2. Experimental

2.1. Chemicals

Levovirin and ICN 10537 (internal standard) (Fig. 1) were supplied by ICN Pharmaceuticals, Inc., Costa Mesa, CA. Acetonitrile (HLPC grade) and water (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA). Triflouroacetic acid was obtained from Sigma (St. Louis, MO).

2.2. Drug administration and plasma sample collection

Male Sprague–Dawley rats ($n = 3$) weighing approximately 300 g were used in the study. Following an overnight fast, rats received an intravenous and oral dose (30 mg/kg) of levovirin (in sterile water) via gavage. Serial blood samples at 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h were collected via cardiac puncture directly into heparinized

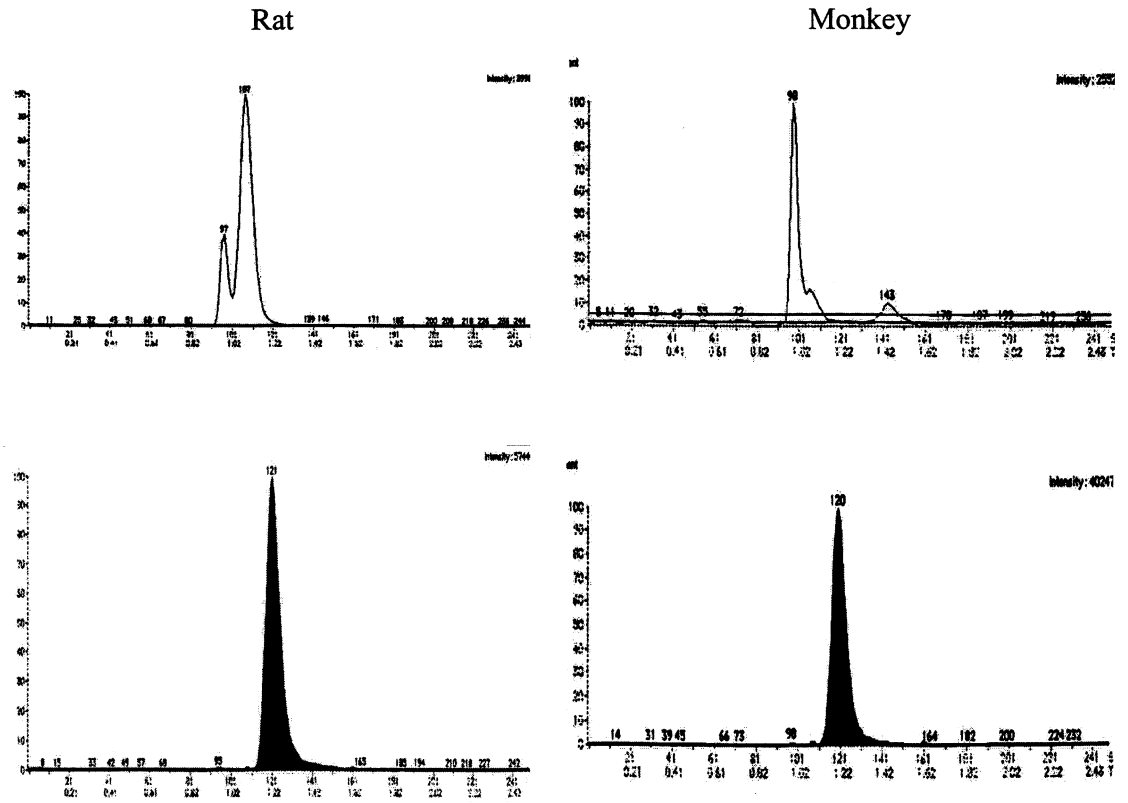


Fig. 2. Typical LC–MS/MS chromatograms of control plasma spiked with internal standard (800 ng/ml).

vacutainer™ tubes following anesthesia with CO_2 and immediately centrifuged for 15 min to harvest plasma. Plasma samples were stored at −20 °C until analyzed.

Male Cynomolgus monkeys ($n = 4$) weighing about 5 kg were used in the study. Following an overnight fast, monkeys received an intravenous or oral dose (10 mg/kg) of levovirin via gavage. Serial blood samples were collected from the indwelling venous cannula and vascular access port directly into heparinized vacutainer™ tubes at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h and immediately centrifuged for 15 min to harvest plasma. Plasma samples were stored at −20 °C.

2.3. Sample preparation

Twenty μl of internal standard (2 μg/ml of acetonitrile:water in a 1:1 ratio), 20 μl of water and 0.425 ml of acetonitrile were added to a 50 μl-aliquot of rat or monkey plasma. The mixture was vortex mixed for 1 min and centrifuged (3000 rpm) for 5 min. The organic layer was transferred to another tube and evaporated to dryness at 45 °C. The residue was reconstituted in 200 μl of HPLC mobile phase (described in Section 2.4) and a 3 μl-aliquot of the mixture was injected into an LC–MS/MS system.

2.4. LC–MS/MS system

The HPLC system consisted of a Shimadzu Model LC-10AD VP pump, a Model SIL-10AD VP autoinjector and a Model SCL-10A VP system controller. Separation was accomplished on an Intertsil Silica column, 50 × 3.0 mm, 5 μm. (Keystone Scientific, Inc.), maintained at 40 °C. The mobile phase consisted of acetonitrile and

0.05% TFA (trifluoroacetic acid) in water (95:5, v/v), delivered at 0.5 ml/min. The effluent from the HPLC system was connected directly to a Perkin–Elmer Sciex Model 3000 MS system equipped with a standard positive electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode. The MS–MS reaction was selected to monitor the $245 \rightarrow 113$ and $259 \rightarrow 128$ transitions for levovirin and internal standard, respectively. Each transition was alternately monitored at a dwell time of 0.5 s.

2.5. Method of evaluation

Precision (% coefficient of variation (CV)) and accuracy (% bias) were determined from the back-calculated concentrations of 9 standard curves prepared in plasma. The lower limit of quantification (LLOQ) was evaluated as the lowest concentration in the standard curve where the %CV (nine replicates) and bias from the back-calculated concentrations were $< 20\%$. Specificity was established by the lack of interference peaks at the retention times of the internal standard and levovirin. The recovery of the internal standard and levovirin was determined using a standard curve set up in a mixture of water–acetonitrile (50:50, v/v). The stability of levovirin (30, 1500 and 3500 ng/ml) was determined for 24 h at room temperature and at the end of 3 freeze–thaw cycles.

2.6. Pharmacokinetic analysis

Plasma concentrations equal to or above the LLOQ were used for pharmacokinetic analysis using a model-independent method. Maximum plasma concentration (C_{max}) and time of C_{max} (T_{max}) were the observed values. The area under the plasma concentration-time curve from time

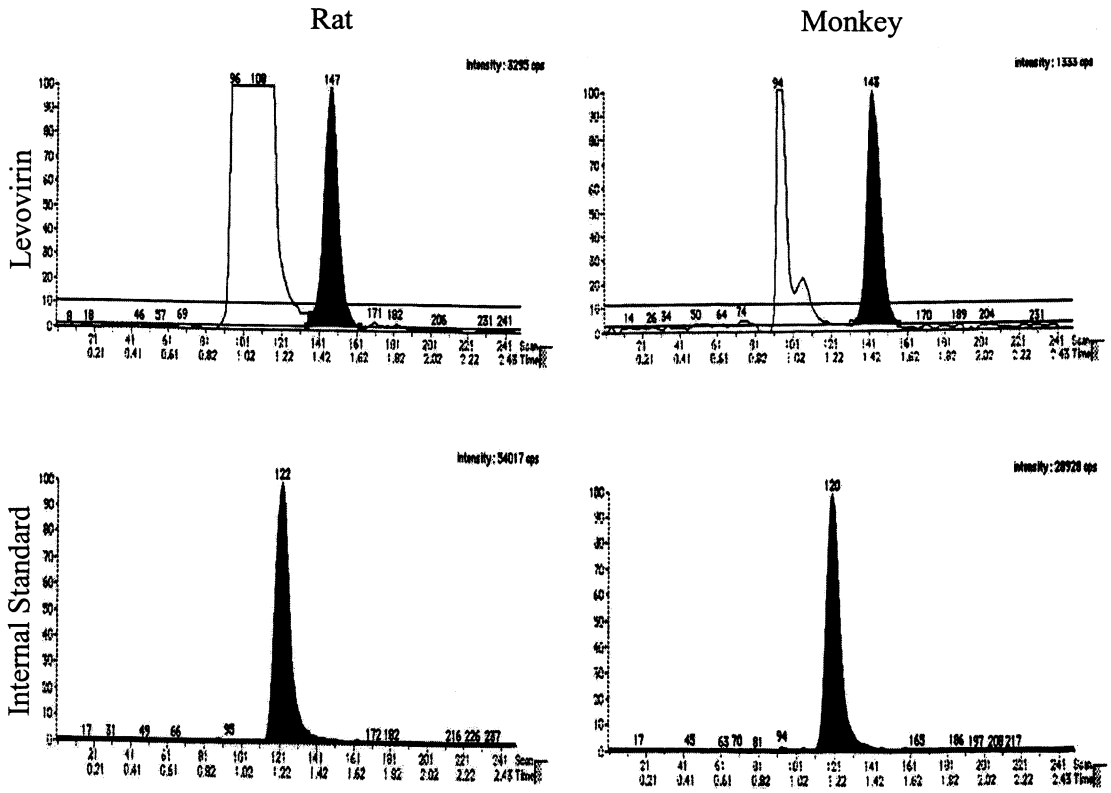


Fig. 3. Typical LC–MS/MS chromatograms of control plasma spiked with 10 ng/ml of levovirin and internal standard (800 ng/ml).

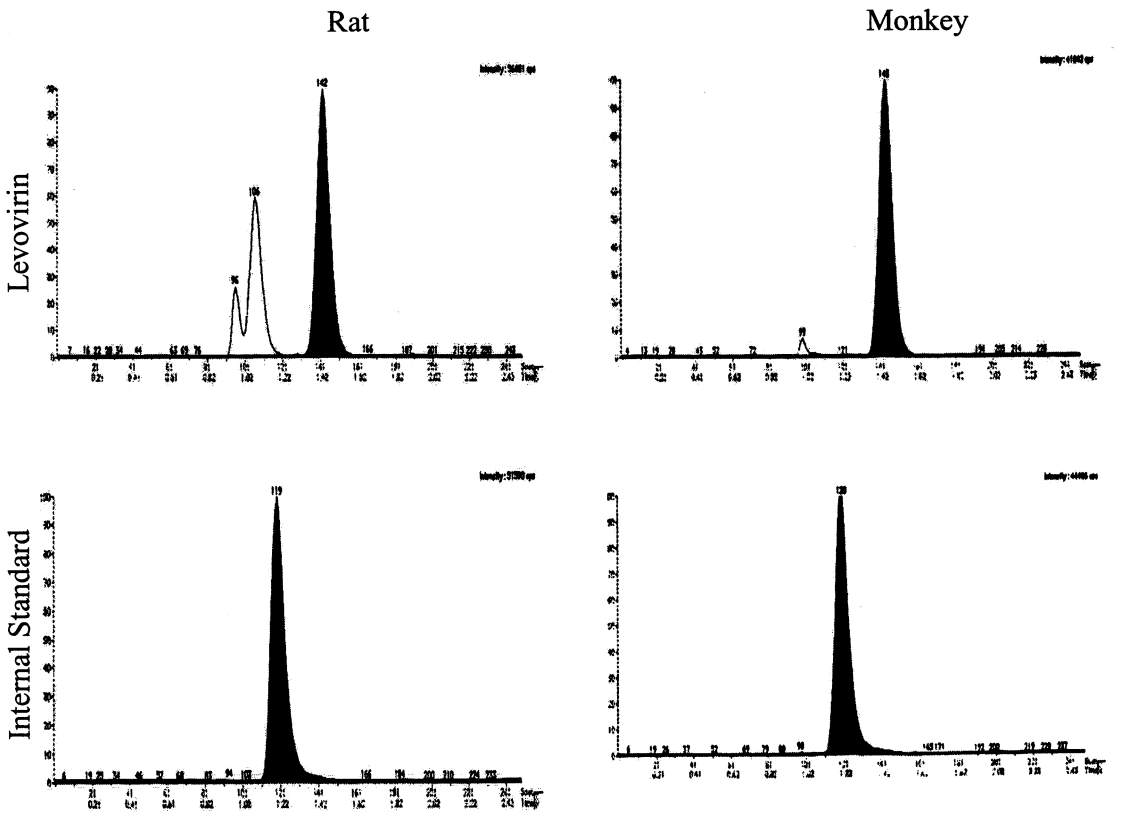


Fig. 4. Typical LC–MS/MS chromatograms of control plasma spiked with 200 ng/ml of levovirin and internal standard (800 ng/ml).

Table 1
Precision and accuracy of levovirin analyses in rat plasma

	Concentration added (ng/ml)	Concentration found (ng/ml) (mean ± S.D.)	CV (%)	Bias (%)
Intra-day	30	27.4	2.2	8.7
	1500	1543	2.4	2.9
	3500	3438	2.1	1.8
Inter-day	30	27.6	2.5	7.9
	1500	1551	2.2	3.4
	3500	3436	1.8	1.8

zero to the time of the final measurable sample [AUC (tf)] were calculated using the linear trapezoidal method. The elimination rate constant (K) was estimated as the negative slope of the regression of log concentration versus time. Half-life ($T_{1/2}$) was calculated by dividing 0.693 by K. Bioavailability was calculated by dividing AUC (tf) obtained after oral dosing by AUC (tf) obtained after intravenous dosing.

3. Results

Typical LC–MS/MS chromatograms for

levovirin and internal standard extracted from plasma are shown in Figs. 2–4. Standard curves were obtained by plotting the ratio of the peak height of levovirin to internal standard against the concentration (10–5000 ng/ml) of levovirin added ($y = 0.00526\ x + 0.00825$ in rat plasma and $y = 0.00488\ x + 0.0129$ in monkey plasma). There was a linear relationship between peak-height ratio and plasma concentration, with a correlation coefficient of 0.9997 in rat plasma and 0.9990 in monkey plasma. The LLOQ was 10 ng/ml, with a small CV (2.9% in rat plasma and 5.0% in monkey plasma) and a small bias (3% in rat plasma and 6% in monkey plasma). Specificity for endogenous interfering peaks was evaluated in 6 lots of rat and Cynomolgus monkey plasma samples obtained from untreated animals. The results demonstrated a lack of analytically-significant interference at the same mass transitions and chromatographic retention times as levovirin or internal standard indicating that the LC–MS/MS analysis for levovirin was specific.

Intra- and inter-day precision and accuracy of the method was evaluated at 30, 1500 and 3500 ng/ml. The results in rat plasma (Table 1) and monkey plasma (Table 2) demonstrated that LC–MS/MS method was accurate (bias $< 9\%$) and reproducible (CV $< 6\%$) in both species.

The recovery of levovirin (30, 1500 and 3500 ng/ml) from rat and monkey plasma was determined to be $> 70\%$, with CV ranging from 2 to 12% (Table 3). Levovirin in rat and monkey plasma was found to be stable at room temperature for 24 h and after three freeze–thaw cycles (Table 4).

Table 2
Precision and accuracy of levovirin analyses in monkey plasma

	Concentration added (ng/ml)	Concentration found (ng/ml) (mean ± S.D.)	CV (%)	Bias (%)
Intra-day	30	30.8	5.3	2.8
	1500	1595	4.9	6.3
	3500	3520	3.4	0.6
Inter-day	30	31.2	4.9	3.9
	1500	1580	4.8	5.3
	3500	3524	2.9	0.7

Table 3
Recovery of levovirin and internal standard from rat and monkey plasma

	Time and conditions	Concentration added (ng/ml)	Concentration found (ng/ml) (mean ± S.D.) ($n = 6$)	CV (%)
Rat plasma	Levovirin	30	71.1 ± 0.06	2.1
		1500	78.7 ± 0.06	7.6
		3500	82.1 ± 0.05	5.6
	Internal standard	30	52.9 ± 0.01	2.3
		1500	52.4 ± 0.04	7.5
		3500	52.8 ± 0.04	7.0
Monkey plasma	Levovirin	30	83.4 ± 0.08	9.7
		1500	82.2 ± 0.10	11.8
		3500	87.0 ± 0.06	7.1
	Internal standard	30	46.9 ± 0.03	5.8
		1500	45.3 ± 0.05	10.3
		3500	47.2 ± 0.03	5.7

Table 4
Stability of levovirin in rat and monkey plasma

	Time and conditions	Concentration added (ng/ml)	Concentration found (ng/ml) (mean ± S.D.) ($n = 6$)	CV (%)
Rat plasma	24 h at room temperature	30	28.5 ± 0.32	1.2
		1500	1520 ± 43.6	2.8
		3500	3437 ± 83.9	2.4
	End of three freeze–thaw cycles	30	26.5 ± 0.32	1.1
		1500	1530 ± 20.0	1.3
		3500	3527 ± 81.4	2.4
Monkey plasma	24 h at room temperature	30	32.0 ± 1.76	5.5
		1500	1530 ± 52.0	3.4
		3500	3390 ± 70.0	2.1
	End of three freeze–thaw cycles	30	30.9 ± 1.99	6.4
		1500	1557 ± 32.1	2.1
		3500	3607 ± 165.0	4.6

Table 5
Pharmacokinetic parameters of levovirin in rats and cynomolgus monkeys

Species	Parameter	Unit	Intravenous	Oral
Rat	Cmax	ng/ml	45 800	2,180
	Tmax	h	0.083	1.5
	AUC	ng h/ml	57 800	11 500
	T1/2	h	1.33	2.75
	Bioavailability	%		20
Monkey	Cmax	ng/ml	28 900	822
	Tmax	h	0.083	3
	AUC	ng h/ml	65 300	12 000
	T1/2	h	3.5	12.2
	Bioavailability	%		18.4

The method was used to analyze plasma samples from rats after intravenous and oral doses of 30 mg/kg and in Cynomolgus monkeys after intravenous and oral doses of 10 mg/kg. After intravenous administration, $T_{1/2}$ was 1.33 h in rats and 3.5 h in monkeys. After oral administration, maximum plasma concentration of levovirin was 2.18 μg/ml in rats and 0.82 μg/ml in monkeys. Bioavailability was 20% in rats and 18.4% in monkeys (Table 5).

4. Discussion

Levovirin is a guanosine nucleoside analogue and the L-enantiomer of ribavirin. In 1983, Austin et al [13] developed a competitive binding radioimmunoassay (RIA) for ribavirin in serum and urine. The assay can detect ribavirin at concentrations as low as 0.25 ng/ml. However, RIA is not specific since anti-ribavirin serum also cross-reacts with the major metabolite of ribavirin. Further-

more, the RIA requires both the synthesis of tritium-labeled compound and the production of specific anti-serum. Therefore, RIA is not the method of choice for the evaluation of levovirin pharmacokinetics. Paroni et al. [14] developed an HPLC method for the determination of ribavirin in human serum and urine with a lower limit of quantitation (LOQ) of 100 ng/ml. It appears that the HPLC method does not have the sufficient sensitivity to properly evaluate the pharmacokinetics of ribavirin or levovirin in animal and man.

Until recently, most analytical methods for the determination of drugs in biological fluids were based on GC or HPLC methods. The introduction of HPLC–API/MS/MS system (HPLC combined with atmospheric pressure ionization tandem mass spectrometry) has provided new opportunities for the rapid development of a more sensitive and specific method. We have, therefore, developed a LC–MS/MS method for the determination of levovirin in rat and monkey plasma.

In the HPLC method, plasma samples were cleaned up with liquid/liquid phase extraction, followed by evaporation to dryness, reconstitution with HPLC mobile phase and HPLC analysis. This sample preparation method is tedious and requires 0.2 ml of plasma. In contrast, the LC–MS/MS method for levovirin as presented in this report is simpler, does not require liquid/liquid phase extraction, and only requires 0.05 ml of plasma. Furthermore, the LC–MS/MS method is specific with a LLOQ of 10 ng/ml. This sensitivity is sufficient to measure plasma concentrations for at least nine half-lives of the C_{max} following intravenous administration at 30 mg/kg (45.8 μg/ml) in rats, and following intravenous administration at 10 mg/kg (28.9 μg/ml) in monkeys. Therefore, major pharmacokinetic parameters such as $T_{1/2}$ and bioavailability were estimated based on plasma concentrations determined in rats and monkeys using the LC–MS/MS method as reported in this communication.

5. Conclusion

The LC–MS/MS method for the determination of levovirin in rat and monkey plasma was validated and was shown to be accurate and reliable over a concentration range of 10–5000 ng/ml with low CV and bias. The method is simple, selective and sensitive and can be used to evaluate the pharmacokinetics of levovirin rats and monkeys.

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