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Journal of Chromatography B, 864 (2008) 9–14
JOURNAL OF CHROMATOGRAPHY B
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A sensitive and specific liquid chromatography–mass spectrometry method for determination of metacavir in rat plasma

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Received 12 August 2007; accepted 25 December 2007
Available online 4 January 2008

Abstract

A sensitive and specific liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) method has been developed and validated for the quantification of metacavir in rat plasma using tinidazole as an internal standard (I.S.). Following ethyl acetate extraction, the analytes were separated on a Shim-pack ODS (4.6 μm, 150 mm × 2.0 mm I.D.) column and analyzed in selected ion monitoring (SIM) mode with a positive ESI interface using the respective $[M+H]^+$ ions, 266 for metacavir and 248 for tinidazole. The method was validated over the concentration range of 1–600 ng/mL for metacavir. Between and within-batch precisions (R.S.D.%) were all within 15% and accuracy (%) ranged from 92.2 to 105.8%. The lower limit of quantification (LLOQ) was 1 ng/mL. The extraction recovery was on average 89.8%. The validated method was used for the pharmacokinetic study of metacavir in rats.

Keywords: Liquid chromatography/mass spectrometry; HBV; Metacavir; Rat plasma; Pharmacokinetics

1. Introduction

Hepatitis B virus (HBV) infection is a major global health concern and the virus infection is a prevalent, worldwide health dilemma. Although chronic HBV infection rates are low in North America and Northern Europe [1], it is endemic in China, Southeast Asia, the Middle East, and sub-Saharan Africa, with up to 10% of these populations affected by chronic HBV infection [1–3]. Asian populations are disproportionately impacted, representing up to 75% of all HBV carriers [1–3]. It is critical that present and future therapies for HBV infection should be safe and effective in Asian populations.

Only a few antiviral drugs are currently under clinical trial for the treatment of persistent HBV infection. Five agents, that is, interferon, lamivudine, adefovir, entecavir and telbivudine are approved for the treatment of chronic HBV infection. Except telbivudine being researched, the other four agents are all associated with therapeutic limitations, including tolerability, resistance, possible nephrotoxicity, and carcinogenicity concerns, respectively [4–8]. There is a continuous demand for new antiviral strategies and new therapies with valid and safe medicines.

Viral polymerase inhibitors that belong to the nucleoside and nucleotide analog family are now widely used as antiviral treatment of chronic hepatitis B. Metacavir, (2-amine-9-(2,3-dideoxy-2,3-dihydro-β-D-arabinofuranosyl)-6-methoxy-9*H*-purine) is a novel synthetic nucleoside for oral administration with potent and specific antiviral activity against HBV. In our previous study in hepatitis B virus (DHBV) infected ducks, DHBV-DNA levels in treated groups were significantly lower than those in control group, which were detected by real-time fluorescence quantitative PCR. This suggests that metacavir suppresses the viral load, thus triggering and an immune response against DHBV infection. There was no evidence of resistant variants after treatment with metacavir. This *in vivo* test showed that metacavir exhibited a potential suppression effect on DHBV replication in models by oral perfusion and intravenous injection. Experimental study on antivirus *in vitro* also indicated that metacavir significantly inhibits the secretion of HBsAg and HBeAg. Since DHBV infection of ducks provides a good model system for HBV

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1570-0232/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2007.12.022

infection in humans [9], this suggests that metacavir may be useful in therapy for HBV infection.

In order to develop an appropriate pharmacokinetics model for metacavir, a sensitive analytical method is necessary to analyze metacavir in biological matrices. There is no report on the development of the method to detect metacavir, although HPLC methods for the quantitation of the other nucleotide analogs (2′,3′-dideoxyadenosine, didanosine, 2′,3′-dideoxyguanosine, etc.) in biological matrices (plasma and cerebrospinal fluid) are available in the literature [10], which uses solid phase extraction technique (SPE) with different cartridges for the extraction of the compound of interest from the biological matrices. However, SPE is generally an expensive and time-consuming process when compared with liquid–liquid extraction (LLE) or protein precipitation techniques. This paper described a rapid, sensitive, accurate and convenient method for the determination of metacavir in rat plasma, which utilized a LLE technique. Moreover, the assay was successfully applied to a pharmacokinetic study in rats.

2. Experimental

2.1. Chemicals and reagents

Metacavir (Fig. 1A, $C_{11}H_{15}N_5O_3$, $M_w = 265.7$) reference standard (99.5% purity) was provided and identified by Changao Pharmaceutical Science & Technology Limited company, Nanjing, China. Tinidazole (Fig. 1B, internal standard, $M_w = 247.2$, 99.5% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol was obtained from Merck (Darmstadt, Germany). Ammonium acetate, ammonia and others chemicals and solvents used were analytical grade. Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA).

2.2. Instrument and analytical conditions

A Shimadzu 2010EV liquid chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan) equipped with an electrospray ionization (ESI) probe and QoQ system (Q-array-octapole-quadrupole mass analyzer) was used in the study. The chromatographic system consisted of an LC-20AD pump, a DGU-20A_3 degasser, a Shimadzu SIL-20AC auto sampler, a CTO-20A column oven and a Shim-pack ODS analytical column (150 mm × 2.0 mm I.D., 4.6 μm, Shimadzu, Kyoto, Japan) with the adoption of a isocratic elution mode using two mobile phases, (A) 0.2 mmol/L ammonium acetate (pH 7.0, adjusted with ammonia) and (B) methanol. The mobile phase was delivered at 0.2 mL/min directly into the ESI source. The column temperature was maintained at 35 °C.

2.3. Mass spectrometric conditions

Both analytes (metacavir and I.S.) were determined in the positive ESI mode. Mass spectrometer conditions were optimized to obtain maximum sensitivity. The curve dissolution line (CDL) temperature was 250 °C and the block temperature was 200 °C. A detector voltage of 1.70 kV and a probe voltage of 4.5 kV were fixed as in the tuning method. The mass spectrometer was equipped with Edwards E2M28 Two stage rotary vacuum pumps (Edwards, UK). Liquid nitrogen (99.995%, from Gas Supplier Center of Nanjing University, China) was used as the nebulizer gas and curtain gas source at 1.5 and 2.0 L/min, respectively. LC/MS Solution Version 3.2 working on Windows XP operating system was used for data processing. The analytes were assayed by quantifying the $[M+H]^+$ ion of metacavir at *m/z* 266.0, and I.S. at *m/z* 248.0. The scans for the analyte and internal standard are shown in Fig. 2.

2.4. Preparation of stock solutions and quality control (QC) samples

The standard stock solutions of 1 mg/mL of metacavir and the internal standard were prepared in methanol, respectively. A series of standard working solutions were obtained by further dilution of the standard stock solution with methanol. Internal standard working solution (10 μg/mL) was prepared by diluting internal standard stock solution with methanol. All solutions were stored at −20 °C. Appropriate amounts of working solution were diluted with drug-free plasma to span a calibration standard range of 1–600 ng/mL. QC samples (1, 6, 60 and 600 ng/mL) were prepared in the same way.

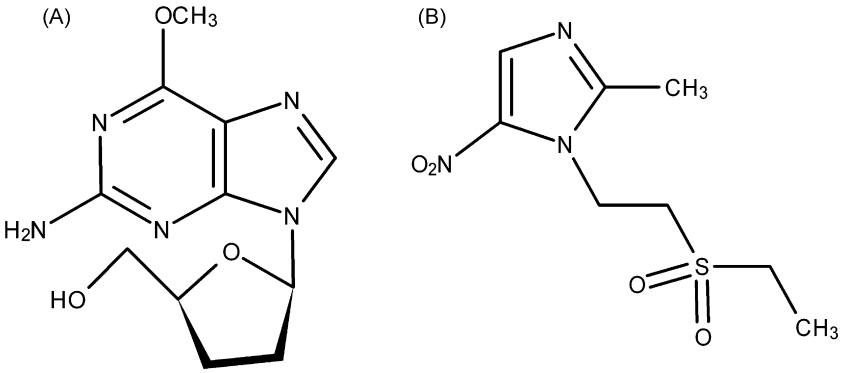

Fig. 1. Chemical structures of metacavir (A) and internal standard tinidazole (B).

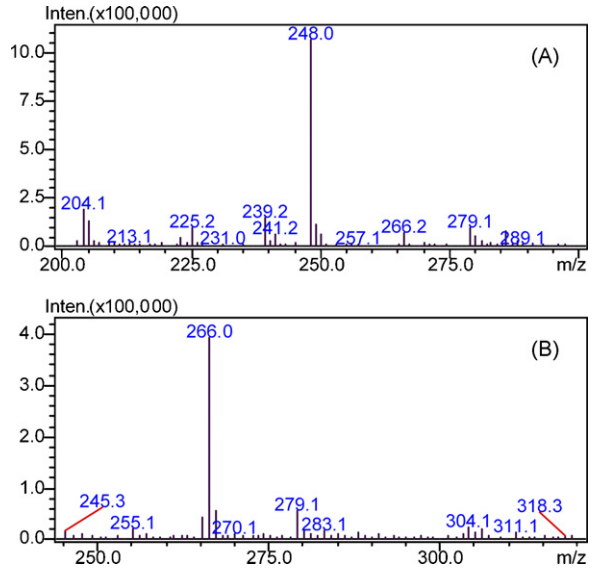

Fig. 2. Positive ion electrospray mass scan spectra of internal standard (A, *m/z* 248.0) and metacavir (B, *m/z* 266.0).

2.5. Sample preparation

Each blood sample was immediately centrifuged after being collected at 4000 rpm for 5 min and plasma was transferred into a clean glass tube to be stored at −20 °C until analysis. Aliquots (100 μL) of the plasma, spiked with 10 μL internal standard working solution, were vortex-mixed (Scientific industries Inc., USA) for 30 s, respectively, and extracted with 3 mL ethyl acetate by a vortex mixing for 3 min. Then the tubes were centrifuged at 4000 rpm for 10 min. The upper organic phase was transferred into clean tubes and evaporated to dryness under a gentle stream of nitrogen gas in a water bath at 37 °C. The residues were then dissolved in 100 μL mobile phase before LC–MS analysis. Aliquots of 10 μL were injected into the LC–MS system.

2.6. Method validation

The method was fully validated for its specificity, matrix effect, linearity, lower limit of quantification (LLOQ), lower limit of detection (LLOD), accuracy, precision and stability [11].

The specificity of the method was evaluated by analyzing blank plasma samples from six rats. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectroscopic conditions and compared with those obtained with an aqueous solution of the analytes at a concentration near the LLOQ.

The matrix effect on the ionization of the analytes was evaluated by comparing the peak areas of the analytes dissolved in the blank sample (the final solution of blank plasma after extraction and dissolution) with that dissolved in the mobile phase. Four different concentration levels of metacavir (1, 6, 60 and 600 ng/mL) and the I.S. were evaluated by analyzing the five samples at each level. The blank plasmas used in this study were six different batches of blank rat plasmas. If the ratio is <85% or >115%, an exogenous matrix effect is implied.

Calibration curves of seven concentrations of metacavir ranging from 1 to 600 ng/mL, which were 1, 2, 6, 20, 60, 200, 600 ng/mL, were extracted and assayed. Peak-area ratios of the analytes to I.S. were calculated and the calibration curve was established by fitting these ratios to the corresponding nominal concentrations by a linear regression method. The LLOQ and the LLOD were considered as the concentrations that produced the signal-to-noise (S/N) of 10/1 and 3/1, respectively.

Assay precision was determined by within- and between-batch relative standard deviation (R.S.D.%) at four concentrations (1, 6, 60 and 600 ng/mL).

The accuracy was determined by comparing the calculated concentration (obtained from the calibration curve) to the theoretical concentration of each sample and expressed as percentage of nominal values.

Recovery experiments were as follow: rat plasma samples were spiked with metacavir at four concentrations (1, 6, 60 and 600 ng/mL) and extracted according to the method described above ($n = 5$). Then 100 ng (10 μL of 10 μg/mL) of I.S. was added to the extracts and the sample was injected into the LC–MS system. The peak-area ratio of metacavir to I.S. was calculated. Standard mixtures of metacavir and I.S. equivalent to the concentration in the rat plasma were directly injected into the LC–MS system and the peak-area ratio of metacavir to I.S. was calculated. The recovery of metacavir evaluated by comparing the peak-area ratios of metacavir: I.S. in the rat plasma extracts to that of the standard mixtures.

The stability was assessed at four concentration levels (1, 6, 60 and 600 ng/mL). Freeze and thaw stability was assessed by using stability QC samples prepared at four concentrations stored at −20 °C and subjected to three freeze–thaw cycles. Short-term stability was assessed by using stability QC samples prepared at four concentrations store at room temperature for a period that exceeded the routine preparation time of the samples (6 h). The long-term stability of metacavir in plasma was assessed at four concentration levels after storage at −20 °C for 4 weeks. The post-preparative stability was assessed during storage in the auto sampler at 4 °C, and performed by repeated injection every 4 h for a period of 24 h. The bench-top stability of metacavir was assessed by removing the rat plasma samples added with metacavir from −20 °C storage, then thawing them by immersion in a 37 °C water bath and putting them on a laboratory temperature for 6 h prior to procession. The stock solution stability of metacavir and I.S. were evaluated at room temperature for 6 h and after storage at 4 °C for 5 weeks.

2.7. Safety considerations

No specific safety precautions should be taken for this method but appropriate measures must be taken for the handling of chemicals and biofluids.

2.8. Pharmacokinetic study

The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Six Sprague–Dawley rats (230–250 g) were obtained from Sino-British Sippr/BK Lab

Animal Ltd. (Shanghai, P.R. China). The rats were maintained in an air-conditioned animal quarter at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 10%, having free access to water, and fed with a laboratory rodent chow (Nanjing, China). The rats were acclimatized to the facilities for 5 days, and then fasted and given free access to water for 12 h prior to experiment. The rats were given single dose of metacavir solution at 25 mg/kg by oral administration. Metacavir solution was prepared by dissolving the powder containing 125 mg of metacavir with 10 mL deionized water. The rats were further fasted for 2 h with free access to water. About 0.25 mL blood samples were collected from the oculi chorioideae vein into heparinized tubes before dosing and at 0.0167, 0.05, 0.083, 0.167, 0.333, 0.5, 1, 1.5, 2, 3 and 4 h after dosing. Plasma was separated by centrifugation at 4000 rpm for 5 min and kept frozen at −20 °C until analysis.

To determine the pharmacokinetic parameters of metacavir, the concentration-time data were analyzed by non-compartmental methods using the DAS Software (ver. 2.0, Medical College of Wannan, China). C_{max} and t_{max} values were obtained directly from the observed concentration *versus* time data. All results were expressed as arithmetic mean ± standard deviation (S.D.).

3. Results and discussion

3.1. LC–ESI-MS method

At first, HPLC was tried to detect metacavir in rat plasma. However, endogenous components could not be separated completely from metacavir. Moreover, the LLOQ in plasma was 50 ng/mL, while many plasma concentrations of metacavir were under LLOQ. Therefore, LC–MS was involved.

There was abundant ionization of metacavir observed under ESI conditions (see Fig. 2). Because of the presence of five nitrogen atoms in metacavir, the intensity of the molecular ion obtained in the positive mode was much stronger than that in the negative mode. When the ion $[M+H]^+$ was selected for determination, the addition of ammonium acetate caused a significant sensitivity increase. So addition of 0.2 mmol/L ammonium acetate to the mobile phase A (adjusted pH to 7.0 with ammonia water) was an important factor for achieving an optimal and stable sensitivity. Comparison was made between the changes caused by modifying the concentrations of ammonium acetate. The best peak shape and ionization were achieved using 0.2 mmol/L ammonium acetate buffer, with pH adjusted to 7.0 with ammonia water. The composition of the mobile phase A can also make the retention of metacavir stable. During the optimization of the method, the signal-to-noise ratio detected at the voltage of 1.7 kV was larger than that at the voltage of 1.6 kV or 1.8 kV. Thus, 1.7 kV was utilized as the detector voltage.

In order to maximize recoveries, different extraction solvents were tested. The recoveries were both high when using methanol to precipitate protein or ethyl acetate as extraction solvent. However, there were more interferences for the determination of metacavir when using methanol to precipitate proteins. No interference existed when determining the free-drug plasma after being extracted by ethyl acetate. Therefore, ethyl acetate was chosen as the extraction solvent. Moreover, 3 mL ethyl acetate and the vortex time, which was 3 min, were two key steps to obtain high recoveries of metacavir and I.S.

3.2. Method validation

3.2.1. Linearity and calibration standard range

The peak-area ratios of metacavir to I.S. in rat plasma varied linearly with concentration over the range: 1, 2, 6, 20, 60, 200, 600 ng/mL. The mean regression equations from five replicate calibration curves on different days were: $R = 0.000503C + 0.000095$, $r = 0.9999$, where R is the peak-area ratio and C the concentration. The standard error of slope was 0.0000037, and the standard error of intercept was 0.000026. The calibration curves were selected based on the analysis of the data by linear regression. The calibration curves of the analytes showed good linearity in the range 1–600 ng/mL for metacavir. The LLOQ was 1 ng/mL. And the LLOD was 0.33 ng/mL which produced a signal-to-noise of 3/1.

3.2.2. Specificity and matrix effect

Typical chromatograms of the blank and spiked plasma are given in Fig. 3, showing the retention times of 5.2 and 3.9 min for metacavir and the I.S., respectively. By comparing Fig. 3A which represented the blank sample with Fig. 3B that came from the spiked rat plasma, no significant interfering peaks were found at the retention times of the analytes. The ratios of the peak area resolved in the blank sample, compared with that resolved by the mobile phase in the matrix effect experiment, were 90, 95, 96 and 99%. The results showed that there was no significant difference between the peak areas of samples prepared from rat plasma and from mobile phase which indicated that no co-eluting invisible compounds significantly influenced the ionization of metacavir. The matrix effect can be neglected in this method.

3.2.3. Accuracy and precision

Table 1 shows a summary of within- and between-batch precision and accuracy at three concentrations. The within-batch precision (expressed as percent relative standard deviation, R.S.D.%) ranged from 0.6 to 12.1% and the within-batch accuracy (expressed as percent of nominal values) ranged from 92.2 to 105.8%. The method showed reproducibility with between-batch precision ranging from 0.9 to 7.0%. The between-batch accuracy ranged from 95.6 to 105.8%. These results indicated that the present method has a satisfactory accuracy, precision and reproducibility.

3.2.4. Recovery

The recoveries of metacavir from rat plasma are shown in Table 2. The mean recoveries were greater than 85% ($n = 5$).

3.2.5. Stability

Stock solutions of metacavir and internal standards were stable when stored at room temperature for 6 h and after storage at 4 °C for 5 weeks. Table 3 displays stability for metacavir under the following conditions: (1) stability of metacavir in rat

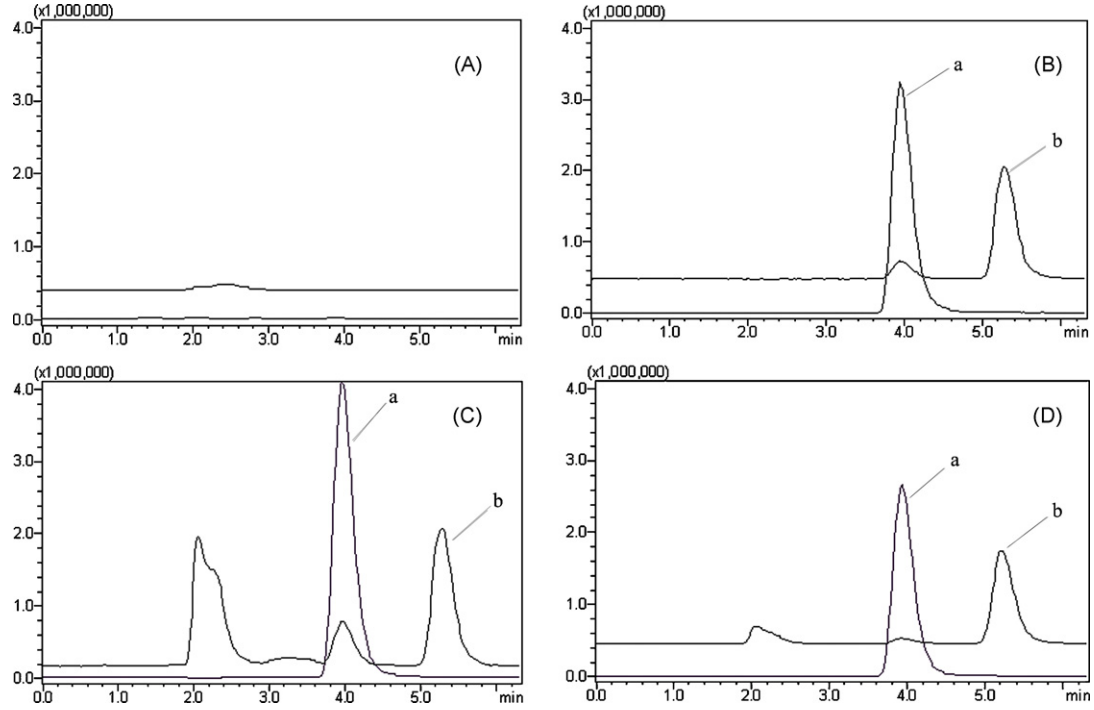

Fig. 3. SIM chromatograms of (A) blank plasma; (B) metacavir and internal standard dissolved in mobile phase; (C) blank plasma spiked with metacavir (200 ng/mL) and internal standard; (D) samples 30 min after oral administration. The retention times of I.S. (a, *m/z* 248.0) and metacavir (b, *m/z* 266.0) were 3.9 min and 5.2 min, respectively.

Table 1
Accuracy and precision for the analysis of metacavir

Spiking plasma concentration (ng/mL)	Within-batch ($n=5$)		Between-batch ($n=5$)	
	Precision (CV, %)	Accuracy (%)	Precision (CV, %)	Accuracy (%)
1	12.1	92.2	7.0	95.6
6	3.6	96.2	3.1	97.7
60	2.7	105.8	1.7	105.8
600	0.6	100.0	0.9	100.2

Table 2
Recovery of metacavir from plasma ($n=5$)

Spiking plasma concentration (ng/mL)	Recovery (mean ± S.D., %)	R.S.D. (%)
1	85.4 ± 4.5	5.3
6	88.2 ± 2.5	2.9
60	86.8 ± 2.4	2.8
600	99.0 ± 2.3	2.4

plasma through three freeze–thaw cycles; (2) short-term stability of metacavir in rat plasma at room temperature for 6 h; (3) long-term stability of metacavir stored at −20 °C for 4 weeks; (4) the post-preparative stability of metacavir during storage in the auto sampler at 4 °C; (5) the bench-top stability of metacavir. As a result, metacavir showed a very good stability under these conditions. The remaining percentages of metacavir were from 104.8 to 115.7%, 95.7 to 110.8%, 103.8 to 106.5%, 98.5 to 102.0% and 95.3 to 105.4%, respectively after freeze–thaw

Table 3
Data showing the stability of metacavir in rat plasma at different QC levels ($n=5$)

Spiking plasma concentration (ng/mL)	Accuracy (mean ± S.D., %)			
	1	6	60	600
Freeze and thaw stability	104.8 ± 5.3	104.1 ± 7.6	106.0 ± 5.0	115.7 ± 6.0
Short-term stability	109.7 ± 4.2	95.7 ± 5.9	110.8 ± 6.9	102.9 ± 7.3
Long-term stability	103.9 ± 4.1	106.5 ± 5.5	104.8 ± 3.7	103.8 ± 4.0
Post-preparative stability	98.5 ± 3.9	102.0 ± 4.0	100.3 ± 2.9	99.0 ± 3.3
Bench-top stability	95.3 ± 5.6	105.4 ± 3.1	102.1 ± 2.8	98.9 ± 6.1

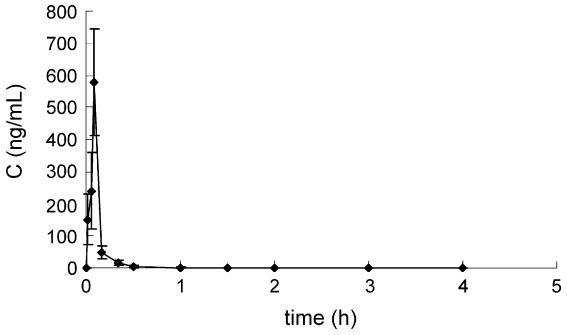

Fig. 4. Mean plasma concentration-time profiles of metacavir in rats after oral administration metacavir of 25 mg/kg, each point and bar represents the mean ± S.D. ($n = 6$).

Table 4
Pharmacokinetic parameters of metacavir following oral administration at a doze of 25 mg/kg, each value represents the mean ± S.D. ($n = 6$)

Pharmacokinetic parameters	Oral administration
C_{max} (ng/mL)	554.2 ± 166.1
T_{max} (h)	0.1 ± 0.0
$t_{1/2}$ (h)	1.5 ± 1.1
MRT (h)	0.6 ± 0.4
$AUC_{0–240}$ (ng h/mL)	48.6 ± 25.9
$AUC_{0–\infty}$ (ng h/mL)	48.7 ± 25.9

cycles, at room temperature for 6 h, stored at −20 °C for 4 weeks, stored at 4 °C for 24 h in the auto sampler and thawed at ambient laboratory temperature for 6 h.

3.2.6. Pharmacokinetic study

This simple, precise and accurate LC–MS method yields satisfactory results for determination of metacavir in rat plasma and has been used successfully in a pharmacokinetic study in rats. The plasma samples were collected at specified time points as described above. After sample extraction and evaporation to dryness, the residue was dissolved in methanol and injected into the LC–MS system. A representative plasma concentration *versus* time profile after oral administration is illustrated in Fig. 4. Based on these results the pharmacokinetic parameters were calculated. The calculated pharmacokinetic parameters are summarized in Table 4. The area under the plasma concentration *versus* time curve from time 0 to 240 min ($AUC_{0–240}$) was 48.6 ± 25.9 ng h/mL. The observed mean maximum plasma concentration (C_{max}) was 554.2 ± 166.1 ng/mL. The mean time to reach the maximum plasma concentration (T_{max}) was 0.1 ± 0.0 h; and the mean elimination half-life ($t_{1/2}$) was 1.5 ± 1.1 h. The concentration of metacavir in rat plasma reached to the maximum more quickly than that of lamivudine ($T_{max} = 2$ h), and metacavir in rat plasma disappeared more rapidly than lamivudine ($t_{1/2} = 2.84$ h). The T_{max} and the $t_{1/2}$ of lamivudine were obtained after its oral or i.v. bolus administration to woodchucks [12]. After its i.v. bolus administration to rhesus monkeys, the mean elimination half-life had shortened greatly. It was 84 min [13]. Perhaps species differences led to the different results in pharmacokinetics. Therefore, various animals should be studied in order to gain overall pharmacokinetics information of metacavir.

4. Conclusion

This paper describes a simple, rapid and specific LC–MS method for investigating pharmacokinetics of metacavir solution in rats. The method results in high sensitivity with a lower limit of quantitation of 1 ng/mL, wide linearity, specificity and no interferences from endogenous substances. It is also an important technique for the further and complete research of the metacavir kinetic behavior *in vivo*. The dosage form of metacavir powder exhibits a fast t_{max} ($t_{max} = 0.1$ h) and a short half-life ($t_{1/2} = 1.5$ h). The mean residence time is about 0.6 h. Further experiments are now being conducted to study the pharmacokinetic profiles of metacavir to gain insights into a safer application of metacavir in the clinics.

Acknowledgement

This study was supported by the National Natural Science Foundation of China (no. 30672500).

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