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Normal-phase liquid chromatography coupled with electrospray ionization mass spectrometry for chiral separation and quantification of clevudine and its enantiomer in human plasma

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ABSTRACT

A new, simple and enantioselective normal-phase liquid chromatography–mass spectrometry method was presented for the quantification of clevudine and its enantiomer in human plasma. A C18 cartridge was used in this method to extract the enantiomers in 200 μL plasma followed by a chiral separation on a cellulose-based LC column with mobile phase consisted of hexane, methanol and ethanol (62:28:10, V/V/V). The eluate was directed to a mass spectrometry through an electrospray ionization interface. A transition of *m/z* 261.0 to *m/z* 126.8 was used for monitoring of clevudine and its enantiomer. This method showed good linearity ($R > 0.997$), precision (<9.6%) and accuracy (within 95.48–105.9%) within a range of 10–1000 ng/mL for the enantiomers and has been applied to the pharmacokinetics study of clevudine capsules in human plasma.

1. Introduction

Clevudine [1-(2-Deoxy-2-fluoro-beta-L-arabinofuranosyl) thymine, L-FMAU, shown in Fig. 1] is a pyrimidine nucleoside analog with potent anti-hepatitis B virus (HBV) activity [1] under clinical trials. It has an enantiomer, D-FMAU [1-(2-Deoxy-2-fluoro-beta-D-arabinofuranosyl) thymine], which showed severe neurologic toxicity in Phase I trials [2]. Therefore, it is important to make sure if there is any biotransformation of clevudine to D-FMAU in vivo after drug administration.

So far, only a few methods [3,4] have been reported on determination of L-FMAU and/or D-FMAU in biological matrix. Furthermore, stereoselective analysis of drug candidates in biological matrix remains a challenge during drug development. It can be carried out in two steps as reported in several literatures [5,6]. Firstly, a non-chiral column was utilized to quantify the total amount of the enantiomers; then a chiral column was applied to separate the enantiomers. The process was laborious and time consuming.

In the present study, a sensitive and reliable LC–MS/MS method for the simultaneous determination of L-FMAU and D-FMAU in human plasma has been developed and validated. This method demonstrated acceptable sensitivity, precision and accuracy. After been extracted by SPE, plasma samples were injected onto a chiral column for separation and then quantified by MS/MS detection. LLOQ of 10 ng/mL was achieved using only 200 μL of plasma. To the best our knowledge, it is the first method for enantioselective determination of FMAU in human plasma.

2. Experimental

2.1. Reagents and chemicals

Clevudine (99.64%) and D-FMAU (99%) were provided by Bukwang Pharm. Co. Ltd. (Korea). Zidovudine, used as internal standard (ISTD), was obtained from Desano Co. (Shanghai, China). HPLC grade hexane and methanol were purchased from Sigma–Aldrich Co. (St. Louis, USA). HPLC grade ethanol was bought from Han-Bang Co. (Anhui, China). Trifluoroacetic acid and formic acid were of analytical grade. Purified water, bought from Wahaha Co. (Hangzhou, China), was used throughout the study.

2.2. Preparation of standard solution

Stock solutions of L-FMAU, D-FMAU and ISTD at 500 μg/mL were prepared in methanol separately. A mixed solution of L-FMAU and D-FMAU was prepared in methanol at 25 μg/mL. Other standard solutions for calibration and QC samples were prepared by serial dilution with 50% methanol from the mixed solution. Standard working solution of ISTD at 2 μg/mL was prepared in water from its stock solution.

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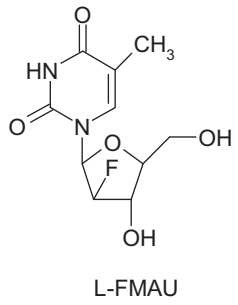

L-FMAU

Fig. 1. Molecular structure of clevudine.

2.3. Preparation of calibration standards and quality control samples

Calibration standards and quality control samples were prepared daily by spiking 10 μL of the appropriate standard solutions to 200 μL blank human plasma. Six calibration standards of L-FMAU and D-FMAU were prepared at 10, 50, 100, 200, 500 and 1000 ng/mL. The lower limit of quantification (LLOQ), low QC (LQC), middle QC (MQC) and high QC (HQC) were prepared at 10, 25, 200 and 800 ng/mL, respectively.

2.4. Instrumentation

The chromatographic system consisted of a Shimadzu (Japan) SCL 10AVP controller, a LC 10ADvp pump, a SILHTC autosampler and a CTO10Avp column oven. An API 3000 Triple-quadrupole tandem mass spectrometer (MDS Sciex, Foster City, CA, USA), equipped with a Turbo Ionspray® interface, was used as detector. The analytical data was processed by Analyst software (Version 1.4.1).

2.5. LC–MS/MS conditions

The chromatographic separation was performed on a Daicel (Osaka, Japan) Chrialcel OJ-H column (250 mm × 4.6 mm, 5 μm particle size). The column temperature was maintained at 30 °C. The mobile phase consisted of hexane, ethanol, methanol and trifluoroacetic (62:28:10:0.02, V/V/V/V). The mobile phase was delivered at flow rate of 0.6 mL/min, and 0.17 mL/min of the elution was split into the mass spectrometry.

A Turbo Ionspray® interface operated in positive mode with ion spray voltage set at 5000 V was used as ionization source. The common parameters, viz., nebulizer gas, curtain gas and collision gas were set at 15, 13 and 6 L/min, respectively. Declusting potential (DP) and collision energy (CE) for enantiomers of FMAU and IS were 27, 21 and 18, 15 V, respectively. Detection of the ions was performed in the multiple reaction monitoring (MRM) modes, monitoring the transition of the *m*/*z* 261.0 precursor ions to the *m*/*z* 126.8 product ions for FMAU enantiomers and *m*/*z* 267.9 precursor ions to the *m*/*z* 127.0 product ions for ISTD. The product ion spectrometry of FMAU is shown in Fig. 2. Quadrupoles Q1 and Q3 were set on unit resolution.

2.6. Sample preparation

To an aliquot of 200 μL plasma, ISTD working solution (2 μg/mL, 200 μL) was added and mixed briefly for about 10 s. Then the mixture was loaded onto a C18 cartridge (200 mg/2.5 mL, Dalian, China), which had been pre-conditioned with 0.5 mL of methanol followed by 0.5 mL water. Then the cartridge was washed with 0.5 mL of 0.1% formic acid solution followed by 0.5 mL of 10% methanol solution. Subsequently, L-FMAU, D-FMAU and ISTD were eluted by 0.5 mL of methanol. The eluate was evaporated to dryness under nitrogen at 50 °C. The residue was dissolved in 200 μL of mobile phase, and a 10 μL aliquot was injected into the LC–MS/MS system.

2.7. Method validation

2.7.1. Specificity and matrix effects

According to the FDA guideline [7], the specificity of the method was evaluated by analyzing human plasma samples from six different sources to investigate the potential interferences at the peak region for analytes and ISTD.

A post-column analyte infusion method was used to judge the matrix effects. A solution containing L-FMAU, D-FMAU and ISTD at 100 ng/mL was continuously infused into the MS/MS at flow rate of 10 μL/min to get constant mass response. The blank plasma extract was injected to the LC–MS/MS system and the changes of mass responses at corresponding peak regions were monitored to evaluate matrix effects.

2.7.2. Linearity

The calibration curves (analyte peak area/ISTD peak area for *Y*-axis and analyte concentration for *X*-axis) were obtained based upon the least square linear regression fit ($y = ax + b$) and a weighting factor of $1/x$. The accuracy of calibration standards was required to be within 100 ± 15% of the nominal concentration, except at LLOQ, which was required to be within 100 ± 20%.

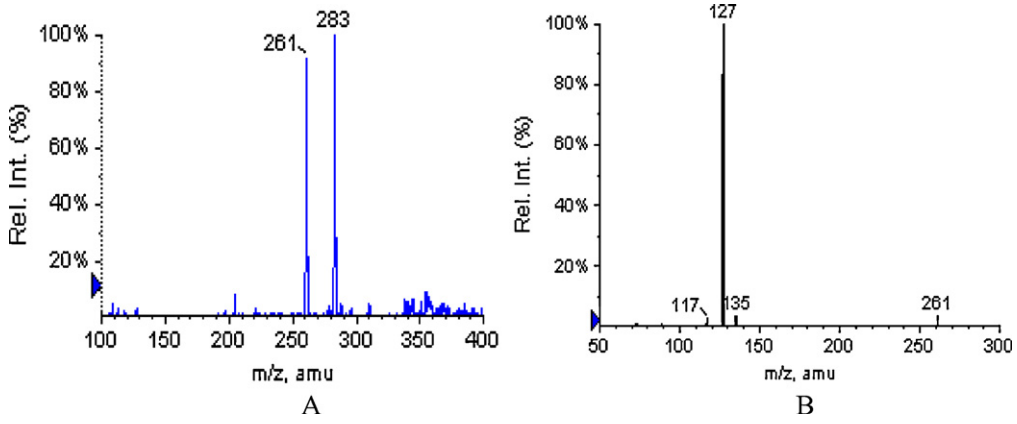

Fig. 2. Parent (A) and product (B) ion spectrum of FMAU.

2.7.3. Accuracy and precision

The precision and accuracy of the method were evaluated by analysis of three separate batches of human plasma samples. Each batch consisted of one set of calibration standards of six levels and six replicates of QC samples at each LQC, MQC and HQC levels. The accuracy and precision were required to be within 100 ± 15% of the nominal value and 15% relative standard deviation (RSD), respectively, for LQC, MQC and HQC samples.

2.7.4. Stability

The stability of L-FMAU, D-FMAU and ISTD in plasma was investigated under a variety of storage and process conditions. Stability evaluations were performed against freshly prepared standard curves. The short-term, long-term stability, freeze–thaw stability, and post-preparative stability were determined, respectively.

2.8. Application

Pharmacokinetics of clevudine capsules in healthy Chinese volunteers was investigated at a single oral dose of 90 mg. Blood samples were collected in heparinized tubes at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 8, 12, 16, 24, and 48 h after drug administration. Plasma samples were separated by centrifugation and stored at −20 °C until assayed.

3. Results and discussion

3.1. Compatibility of normal phase and MS

The normal-phase liquid chromatography systems (e.g., mixture of alcohol and hexane) are generally considered as not compatible with ESI or APCI ionization techniques due to potential hazard and poor ionizability [8,9]. During method development, enhancing the polar organic-phase proportion in mobile phase was adopted to reduce the risk of the potential hazard and enhance ionization of the analyte. Under the optimized condition, neither explosion nor relevant phenomenon happened. Meanwhile, good ionization efficiency was achieved without post-column addition of polar solvents.

The two main ionization sources, APCI and ESI, were compared during method development, and ESI was chosen for its better ionization efficiency. Under ESI mode, the mass response of FMAU in acidic mobile phase was much higher (about one order of magnitude) than that in neutral mobile phase. Formic acid, acetic acid and TFA were investigated, respectively, and TFA was the best acid additives because of higher mass response and better peak shape.

3.2. Chiral separation

Because D-FMAU and L-FMAU shared the same ion transition channel, baseline resolution of the two isomers on column was necessary. A Daicel Chrialcel® OJ-H column, operated in normal phase, was selected for chiral separation. The chromatographic conditions, especially the mobile composition were optimized through several trials to achieve good resolution and symmetric peak shape. The retention of the analyte on polysaccharide CPS became shorter when the polarity of mobile phase was increased. Ethanol is generally recommended as polar content, however, in this method a mixture of ethanol and methanol was used, because it can get good separation, suitable system pressure and run time. A more important reason is that this mixture can be compatible with MS/MS. TFA was used as acid additive to improve separation and peak symmetry.

3.3. Sample preparation

Compared with conventional columns, chiral separation columns were susceptible to biological matrix, which may reduce column efficacy or even damage columns. Therefore, plasma sample preparation was another key process in this method. It was difficult to extract clevudine from plasma by liquid–liquid extraction because of its insolubility in organic solvents (e.g., ether, hexane). Protein precipitation method was not effective for removing residual matrix components, so solid phase extraction method was an optimum choice. In order to remove more matrix components and improve extraction recovery, 0.1% formic acid solution was chosen to wash the SPE columns, instead of pure water. 0.1% formic acid and 10% methanol solution in this method can remove more matrix components with less analytes loss. No significant damages or changes were observed on the chiral column after more than one hundred samples have been injected.

3.4. Method validation

3.4.1. Specificity and matrix effects

Typical chromatographs are shown in Fig. 3. Retention times of D-FMAU, L-FMAU and ISTD were 9.7 min, 10.9 min, and 11.1 min, respectively. Resolution value between D-FMAU and L-FMAU was 2.5. No interference peaks were observed for D-FMAU, L-FMAU and ISTD from any of six lots of blank human plasma.

Matrix components, which are not observed in the LC–MS/MS chromatography, can have a detrimental effect on the analysis [10]. The post-column infusion method identified chromatographic regions most likely to experience matrix effects. The results showed that FMAU and ISTD were separated from matrix window, their mass responses nearly constant at corresponding peak regions (shown in Fig. 4), so the influence of matrix was negligible.

3.4.2. Linearity

The linearity was evaluated based on the average of six calibrators analyzed in three batches. Typical equations were $y = 0.000768x - 0.00135$ and $y = 0.000772x + 0.00146$ for L-FMAU and D-FMAU, respectively. Acceptable linearity was achieved in the range of 10–1000 ng/mL for both analyte. The coefficients of determination (r) for L-FMAU and D-FMAU were greater than 0.997 in all validated batches. Lower limit of quantification (LLOQ) was 10 ng/mL and the lower limit of detection (LLOD) was 5 ng/mL with S/N no less than 3.

3.4.3. Precision and accuracy

The precision of L-FMAU was less than 9.6% RSD and the accuracy was within the range of 95.48–104.4% over the three concentration levels evaluated in all the three batches (Table 1). Similarly, the precision and accuracy for D-FMAU were within 9.6% RSD and 95.62–105.9% (Table 1), respectively. The inter-batch precision and accuracy of QC samples at different levels are also shown in Table 1. These results indicated that excellent precision and accuracy can be achieved for this assay. The accuracy and precision of L-FMAU at LLOQ was 103.4 and 13.5%, while 102.0% and 9.6% for D-FMAU.

3.4.4. Stability

No stability issue was observed from those experiments described in Section 2.7.4. The results of stability test are shown in Table 2, which indicated that L-FMAU and D-FMAU were stable in plasma at room temperature for 6 h, at −20 °C for 90 days, after three freeze–thaw cycles, at 2–8 °C for 24 h post process.

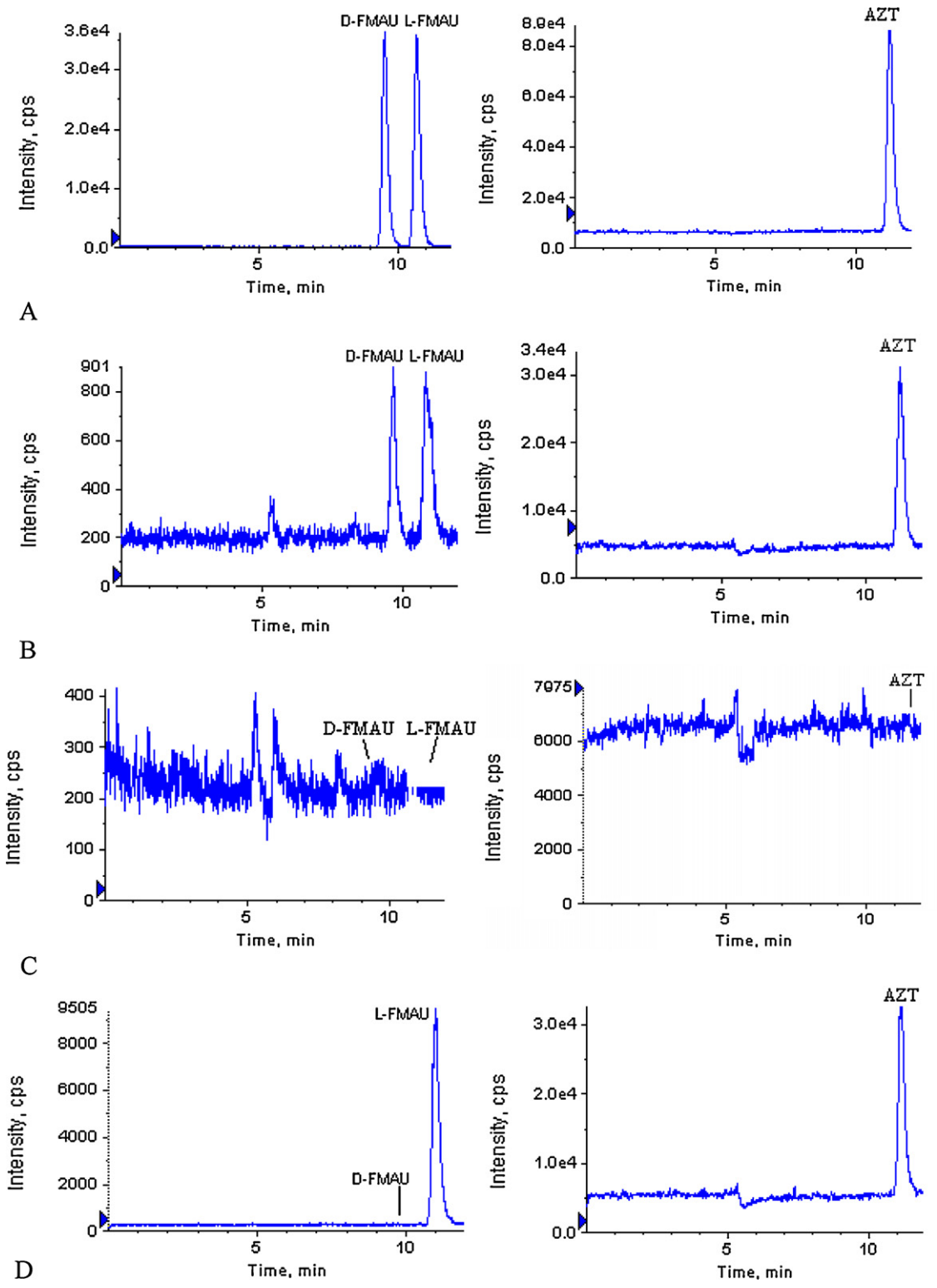

Fig. 3. Typical chromatograms: (A) L-FMAU, D-FMAU and IS standard solution (500 ng/mL); (B) plasma sample spiked with L-FMAU, D-FMAU and IS at the LLOQ (10 ng/mL) (C) drug free plasma; (D) plasma sample post dose 4 h from subject 28#.

3.5. Internal standard

A good internal standard should offset the deviations throughout the methods, including sample preparation, chromatographic elution and mass spectrometric detection. Stable isotopes of the analytes are usually the best candidates. However, isotope internal standards are not always available easily. Some compounds were investigated and Zidovudine, a commercially available and structurally similar compound, was chosen. The validation results indicated that ISTD offset most deviations in the process.

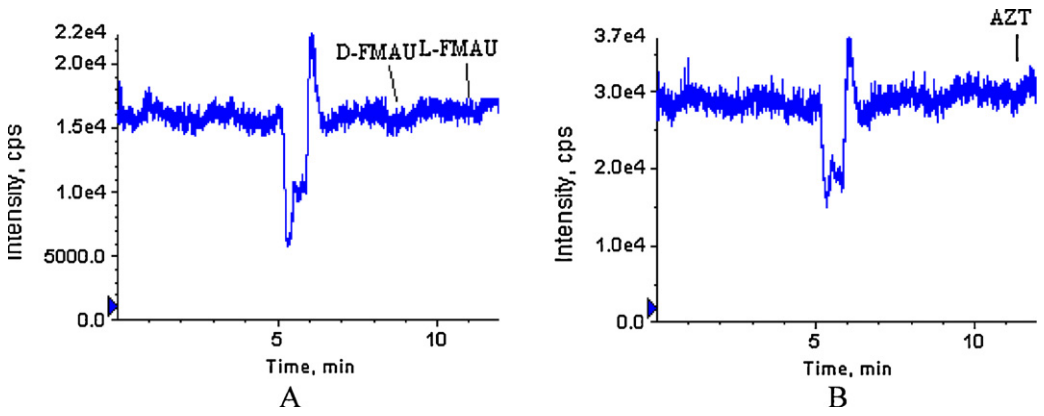

Fig. 4. Matrix effects influence on the mass spectrometry response of FMAU (A) and AZT (B).

Table 1
Precision and accuracy of quality control samples of L-FMAU and D-FMAU.

Nominal concentration (ng/mL)	Batch	L-FMAU		D-FMAU	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Intra-day variation (six replicates at each concentration)					
	1	105.2	6.2	104.4	4.7
800	2	95.62	3.8	96.14	2.9
	3	99.75	4.0	100.9	3.5
	1	101.1	7.0	106.8	2.6
200	2	95.73	5.1	95.48	4.5
	3	96.33	5.8	98.91	6.5
	1	104.3	5.1	102.9	9.6
25	2	105.9	4.9	101.9	5.3
	3	97.27	9.0	97.05	5.7
Inter-day variation (Eighteen replicates at each concentration)					
800		100.2	6.1	100.5	5.0
200		97.73	6.2	100.4	6.6
25		102.5	7.1	100.6	7.3

3.6. Application

The validated method was applied to analyze plasma samples from three subjects after oral administration of 90 mg clevudine capsules. This method was sensitive enough to monitor the plasma concentration of L-FMAU up to 48 h. The mean plasma concentration–time profile of L-FMAU is shown in Fig. 5. No D-FMAU, above limit of detection, was detected in all tested samples, while the mean maximum plasma concentration of L-FMAU was 384.7 ng/mL. It showed that no biotransformation of L-FMAU to D-FMAU occurred in human body.

Table 2
Stability of quality control samples of L-FMAU and D-FMAU.

Nominal concentration (ng/mL)	L-FMAU		D-FMAU	
	Accuracy (%)	RSD (% $n = 3$)	Accuracy (%)	RSD (% $n = 3$)
Short-term stability (6 h at room temperature)				
800	105.9	6.3	104.1	5.1
200	108.5	7.4	106.2	8.0
25	102.9	10.1	99.80	5.5
Long-term stability (90 d at −20 °C)				
800	104.7	8.8	103.2	6.2
200	103.2	6.0	102.1	5.5
25	105.8	5.0	104.6	6
Freeze-thaw stability				
800	107.2	5.5	105.2	4.2
200	107.4	3.0	106.1	2.3
25	93.34	8.0	98.21	6.0
Post-preparative stability (24 h at 2–8 °C)				
800	97.34	2.2	99.80	3.1
200	100.9	2.2	102.1	2.5
25	106.2	3.6	104.2	2.4

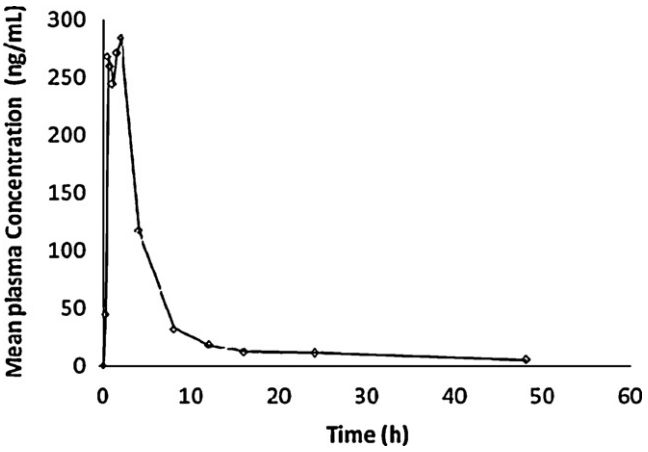

Fig. 5. Mean plasma concentration–time profile of L-FMAU after single oral dose of 90 mg clevudine.

4. Conclusions

A normal phase liquid chromatography coupled with tandem mass spectrometry assay was validated for chiral separation and quantification of L-FMAU and D-FMAU in human plasma. This method was successfully applied to analyze plasma sample from pharmacokinetics study of L-FMAU.

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