Determination of Adefovir by LC–ESI-MS–MS and Its Application to a Pharmacokinetic Study in Healthy Chinese Volunteers

Jianjun Zou[1], Bin Di[2], Junren Zhang[3], Li Dai[2], Li Ding[2], Yubing Zhu[1], Hongwei Fan[1], and **DaWei Xiao[1,*].**

[1]Department of Cardiovascular and thoracic Surgery, the First Affiliated Hospital, Soochow University, Suzhou, China; [2]College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China; [3]School of Pharmacy, China Pharmaceutical University, Nanjing, China; [4]Department of Clinical Pharmacology, Nanjing First Hospital of Nanjing Medical University, Nanjing, China

Abstract

A selective and sensitive high-performance liquid chromatography-electrospray-tandem mass spectrometry method (HPLC–ESI-MS–MS) has been developed for the determination of adefovir in human plasma using adenine (PMPA) as an internal standard. After protein precipitation with methanol, the plasma sample was separated by HPLC on a reversed-phase XTerra MS/MS C_{18} column (100 mm × 2.1 mm i.d., 3.5 mm) with a mobile phase of methanol–water (20:80, v/v). Standard curves were linear (r^2 = 0.9962) over the concentration range of 0.20–100 ng/mL and had acceptable accuracy and precision. The intra- and inter-batch precisions were within 11.30%. The lower limit of quantification (LLOQ) was 0.20 ng/mL. The validated HPLC–ESI-MS–MS method has been used successfully to study the pharmacokinetics of adefovir in healthy Chinese volunteers. The following pharmacokinetic parameters were elucidated after administering a single dose of 10 mg, 20 mg, and 30 mg of adefovir dipivoxil. Peak plasma concentrations (C_{max}) were (26.6 ± 6.1), (55.7 ± 16.2), and (70.2 ± 11.8) ng/mL, respectively; time to C_{max} (T_{max}) were (1.5 ± 0.6), (1.6 ± 0.7), and (1.8 ± 0.6) h, respectively; the area under the plasma concentration versus time curve from time 0 h to 36 h ($AUC_{0–36}$) were (184.5 ± 25.2), (379.3 ± 61.8) and (556.5 ± 86.7) ng/mL, respectively; mean residence times (MRT) were (8.9 ± 0.9), (9.0 ± 1.0), and (8.9 ± 1.0) h, respectively; and the elimination half-life ($t_{1/2}$) were (8.0 ± 0.9), (7.5 ± 0.8), and (7.5 ± 0.9) h, respectively. The pharmacokinetic parameters can provide some information for clinical medication.

Introduction

Adefovir dipivoxil is a diester prodrug of the nucleotide analogue, adefovir (Figure 1), which is a broad-spectrum antiviral agent with activity against herpes viruses. Pharmacological studies show it effectively inhibits both Hepatitis B virus (HBV) replication and disease activity in patients with chronic Hepatitis B (1–3). Adefovir dipivoxilis is spontaneously hydrolyzed to adefovir by cellular esterases (4). Adefovir is the primary circulating metabolite identified after oral adefovir dipivoxil administration (5). The therapeutic daily oral dose of 10 mg adefovir dipivoxil is given, which results in lower plasma concentrations in the elimination phase, and hence a sensitive analytical method is needed for its determination in plasma. Several methods for the quantification of adefovir in plasma have been reported. Sparidans RW (5) established a high-performance liquid performance (HPLC)–fluorescence detection method to determine the derivative of adefovir in plasma, and the lower limit of quantification (LLOQ) of adefovir was 20 ng/mL. The sample preparation and extraction procedure of these methods were time consuming. Both Z.Y. Meng et al. (6) and L.Y. Zhao (7) reported a HPLC–electrospray-tandem mass spectrometry (ESI-MS–MS) method for the analysis of adefovir in monkey plasma with LLOQ of 20 ng/mL. A liquid chromatography–tandem mass spectrometry (LC–MS–MS) method (8) has been reported for the determination of adefovir in human plasma with LLOQ of 1.5 ng/mL. Y. Liu (9) reported an LC–MS–MS method to determine adefovir in human serum with an LLOQ of 0.10 ng/mL. The plasma samples

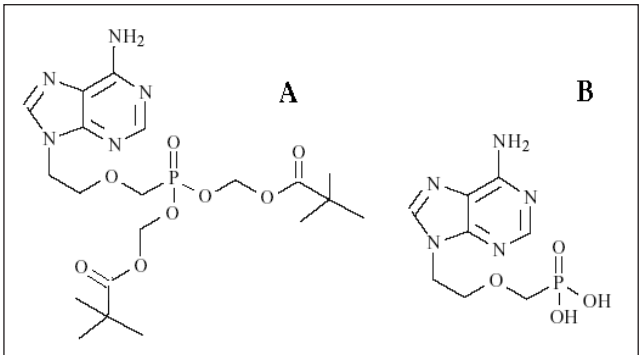

Figure 1. Chemical structures of Adefovir dipivoxil, A; Adefovir, B; and PMPA, C.

Author to whom correspondence should be sent: email zoujianjun100@126.com, dawei_xx@hotmail.com.

were treated by a complex solid-phase extraction (SPE) procedure. X.Y. Chen (10) described a LC–MS–MS method to determine adefovir in human serum with an LLOQ of 0.25 ng/mL. The purpose of this paper was to explore a higher selectivity and sensitivity of an MS–MS system with an ESI for the development and validation of a robust reversed-phase LC–MS–MS method for the quantification of adefovir in human plasma.

Experimental

Chemicals and reagents

Adefovir standard reference (Figure 1, purity: 99.6%, lot no. 20050912) and adefovir dipivoxil capsules (10 mg/capsule, lot no. 20051201) were kindly provided by Dehua Pharmaceutical Limited (ShangHai, China). The internal standard (9-[(R)-2-(phosphonomethoxy)propyl]adenine (PMPA) Figure 1) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (purity: 99.4%, lot no. 100309-200001; NICBP, Beijing, China); HPLC-grade methanol was purchased from Tedia Company, Inc. (Fairfield, Ohio). Water was deionized and purified using a Milli-Q system (Millipore, Bedford, MA) and was used to prepare all aqueous solutions. Other chemicals and reagents were of analytical-grade. Drug-free and drug-containing plasma were taken from the volunteers. Plasma was stored at –40°C until assayed.

Instrumentation and operating conditions

The HPLC system consisted of a Surveyor MS pump and a Surveyor autosampler (Thermo Scientific, Waltham, MA). The column was an XTerra MS–MS C_{18} column (100 mm × 2.1 mm i.d., 3.5 µm) and was operated at 25°C. The mobile phase consisted of methanol–water (20:80, v/v) and was set at a flow rate of 0.2 mL/min, and the injection volume was 20 µL. Separation was conducted under isocratic conditions, and the total running time was no more than 7.0 min. The autosampler was controlled at 8°C. Mass spectrometric detection was performed using a Surveyor LC-TSQ Quantum Ultra AM tandem mass spectrometer. After establishing the final conditions for the chromatographic analysis of adefovir, the detector interface and mass spectrometer were systematically optimized to maximize the response for the adefovir $[M-H]^-$ ion with detection in the selected reaction monitoring (SRM) mode, ESI-MS–MS was performed in the negative mode: capillary temperature was 350ºC, spray voltage, tube lens offset, source CID, and collision energy were 4000, 101, 2, and 25 V, respectively; Sheath gas, ion sweep gas, and aux gas pressure were 40, 1, and 24 psi, respectively. The gas used was of high purity; collision pressure was 0.8 torr; scan time was 0.2 s; SRM was used to quantify adefovir 272.0 *m/z* $[M-H]^-$, daughter ion 134.0 *m/z*; and PMPA 286.3 *m/z* $[M-H]^-$, daughter ion 134.0 *m/z* as shown in Figure 2 and Figure 3. The two pairs of ions were monitored simultaneously within the analytical procedure. The quantification was performed via peak area ratio (peak area of analyte/peak area of PMPA). Data acquisition and processing were accomplished using the Xcalibur workstation for Finnigan TSQ Quantum system.

Preparation of standard solutions

Primary stock solutions adefovir and PMPA were prepared at 0.5 mg/mL in methanol and stored at –40°C. Standard solutions of adefovir were prepared at concentrations of 100 µg/mL,10 µg/mL, 1 µg/mL, 100 ng/mL, 10 ng/mL, and 1 ng/mL by diluting the primary stock solution with methanol in separate 10-mL volumetric flasks. A solution containing 1 µg/mL PMPA was also prepared by further diluting the primary stock solution of PMPA with methanol. All the solutions were stored at –40ºC.

Sample preparation

Fifty microliters of 100 ng/mL PMPA solution was added to a 0.20 mL aliquot of plasma. The sample mixture was deproteinized with 0.4 mL of methanol, and the precipitate was removed by centrifugation at 16,000 rpm for 5 min. A 300 µL aliquot of the supernatants was transferred to another tube and evaporated to dryness under a stream of nitrogen in a water bath

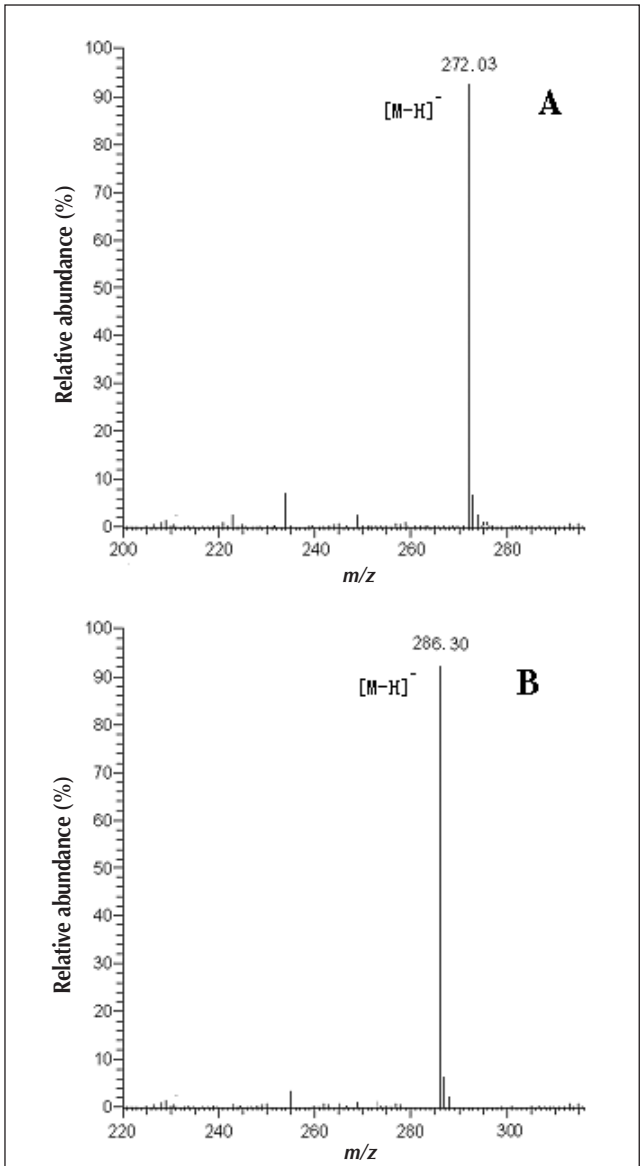

Figure 2. Negative ion electrospray mass spectrum plot of parent ions for adefovir, A; and PMPA, B; by continuous scanning.

of 50ºC. The residue was dissolved in 100 µL of the mobile phase and vortex-mixed. A 20 µL aliquot of the sample solution was subjected to LC–MS–MS system.

Preparation of quality control samples

Quality control (QC) samples were prepared in 0.2 mL of blank plasma at concentrations of 0.3, 1.5, 7.5, and 37.5 ng/mL for adefovir and stored at –40°C. QC samples were analyzed with processed test samples at intervals in each run. The results of the QC samples provided the basis of accepting or rejecting the run.

Method validation

The method validation assays were carried out according to the Food and Drug Administration document (11). Analyses of the blank samples of the healthy subjects' blank plasma were obtained from six sources, and each blank sample was tested for the visible interference. In order to evaluate the matrix effect on the ionization of analytes, four different concentration levels of adefovir standard solution were added to the dried extracts of the 0.2 mL blank sample. The residues were dissolved in 200 µL of mobile phase. The same concentration levels of adefovir were vortex-mixed with the same volume of the mobile phase. The matrix effect on internal standard was evaluated using the same method.

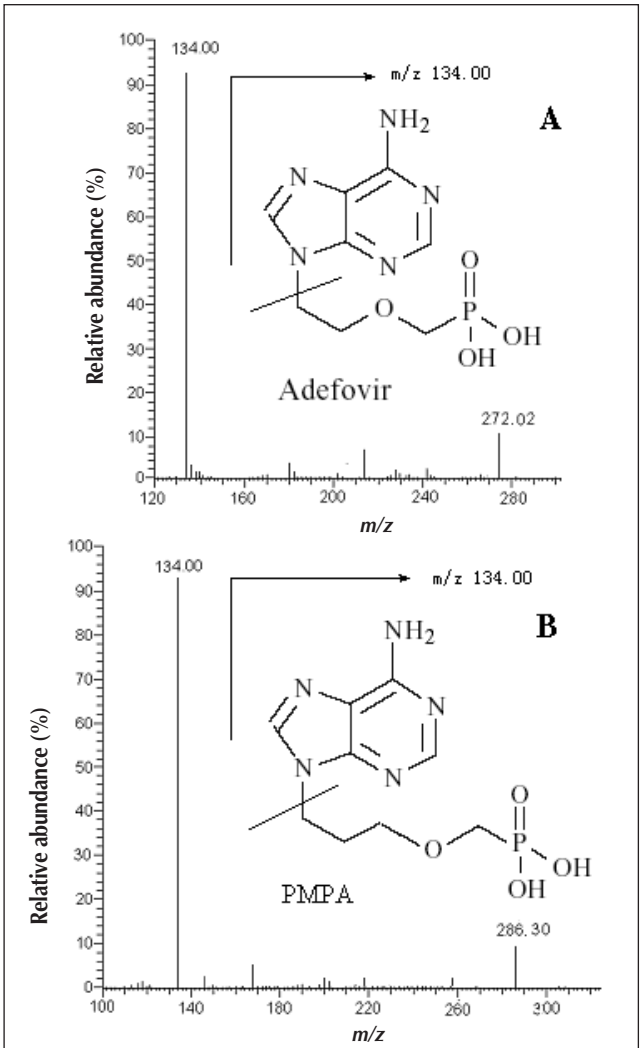

Figure 3. Product ion mass spectra of adefovir, A; and PMPA, B.

Standard curves were prepared by determining the best-fit of peak area ratios (peak area of analyte/peak area of PMPA) versus concentration and fitted to the equation $R = bC + a$ by weighed least-squares regression with a weighting factor of $1/C^2$. Standard curves of nine concentrations of adefovir ranging 0.20–100 ng/mL were prepared and assayed. The LLOQ were determined as the concentrations with a signal-to-noise ratio of 10, respectively. Precision and accuracy were assessed by determining QC plasma samples at four concentration levels on the three different validation days. The concentration of each sample was determined using standard curve prepared and analyzed on the same day. The extraction recovery of adefovir was determined by comparing the adefovir/PMPA peak area ratios (R_1) obtained from extracted plasma samples with those (R_2) from standard solutions at the same concentration. This procedure was repeated for the four different concentrations of 0.3, 1.5, 7.5, and 37.5 ng/mL ($n = 5$).

The long-term stability of adefovir in human plasma was evaluated by keeping QC plasma samples at low temperature (–40°C) for 30 days. The short-term temperature stability of adefovir was assessed by keeping QC plasma samples at room temperature (20–30°C)for 8 h. The post-preparative stability was determined by placing QC samples under the autosampler conditions (8°C) for 24 h. The dried extract stability was tested by analyzing QC plasma samples kept in a deep freezer at –40°C for 48 h. The freeze-and-thaw stability was also tested by analyzing QC plasma samples undergoing three freeze (–40°C)-thaw (room temperature) cycles on consecutive days.

Pharmacokinetics Study Design and Pharmacokinetic Analysis

Subjects

Pharmacokinetics study was conducted on healthy Chinese volunteers according to the revised Declaration of Helsinki for biomedical research involving human subjects and the current Good Clinical Practice standard (GCP).

The study was performed on healthy male and female volunteers, 18–25 years of age, after they had been informed of the purpose, protocol, and risk involved in the study. All subjects gave written consent, and the ethics committee of the First Affiliated Hospital of NanJing Medical University (NanJing, China) approved the protocol. Subjects were screened by way of a physical examination and laboratory tests, which included hematology, blood biochemistry, and urine analyses. No volunteers had a history or evidence of a renal, gastrointestinal, hepatic, or hematologic abnormality; or any acute or chronic disease; or an allergy to any drugs.

All subjects were instructed to abstain from some food and beverages (like chocolates, tea, coffee or other caffeinated drinks, cigarettes, tobacco, and alcoholic products) for 48 h prior to dose administration and throughout their stay at the clinical facility. During the 24 h period after drug administra-

tion, no strenuous physical or mental activity was permitted. All subjects were hospitalized at 8:00 p.m. for one day before this study and were in fasting state for at least 10 h before dosing. They were not allowed to drink water from 1 h pre-dose to 2 h post-dose; from thereafter, free access to drinking water was allowed.

Study design of single doses

Thirty-six healthy volunteers, 18 males and 18 females, are randomly divided into three groups, (group A, B, and C). Each group was made up of six males and six females. After an overnight fasting (10 h), group A , B, and C were administered a single dose of adefovir dipivoxil (10 mg, 20 mg, and 30 mg) with 250 mL of water, respectively. A total of 15 heparinized blood samples were collected during each period. Venous blood samples were withdrawn via an indwelling cannula at pre-dose (0 h) and 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, and 36 h after dosing. The samples were collected in pre-labeled tubes containing heparin as the anticoagulant and centrifuged at 4000 rpm for 10 min at 25ºC. Plasma was collected in pre-labeled sample collection tubes. The samples were stored in the deep freezer at –40ºC until analyzed.

Study design of multidoses

Group A received 10 mg of adefovir dipivoxil with 250 mL of water at 8:00 a.m. and at 8:00 p.m. for 13 consecutive oral doses at 12 h intervals. On day 5, day 6, and day 7, 5 mL of venous blood before every dosing at 8:00 a.m. was drawn to observe the minimum value of steady plasma-drug concentration. On day 7, the procedure was like that mentioned previously.

Pharmacokinetic analysis

The pharmacokinetic parameters, namely maximum plasma concentration (C_{max}), time point of maximum plasma concentration (T_{max}), area under the plasma concentration–time curve from 0 h to the last measurable concentration (AUC_{0-t}), area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$), apparent volume of distribution (V/F), MRT, and half-life of drug elimination during the terminal phase ($t_{1/2}$) were calculated using Drug and Statistics Software (Version 2.0, Chinese).

Results and Discussion

Sample preparation and method development

Sample preparation is usually required for the determination of pharmaceuticals in biological samples owing to complex matrices in order to remove possibly interfering matrix components and increase the selectivity and sensitivity. Liquid–liquid extraction (LLE) is a widely adopted method and often achieves satisfactory extraction recoveries of analytes from biological samples (12–14), but adefovir is a highly polar component, which can hardly be extracted by LLE. Solid phase extraction (SPE) is necessary and important because this technique could not only purify but also concentrate the sample (9). But the sample preparation and extraction procedure of these methods are time-consuming. In the present work, protein precipitation was used for the extraction of the adefovir from human plasma, which produced a clean chromatogram for a drug-free plasma sample and offered satisfactory extraction recoveries for the analyte. An XTerra MS–MS C_{18} column (100mm × 2.1 mm i.d., 3.5 µm) column was used for the chromatographic separation. In optimizing the chromatographic conditions, the concentration and pH of the ammonium acetate buffer were investigated. The chromatographic peaks were not improved when we increased or decreased the concentration of ammonium acetate buffer, and the addition of 1% formic acid decreases the response of adefovir. Thus, a mix of methanol–water (20:80, v/v) was finally adopted in the mobile phase, which provided symmetric peak shapes of the analytes and the internal standard. The representative chromatograms were shown in Figure 4. Typical retention times were about 5.2 min for adefovir and 6.0 min for PMPA.

Selection of PMPA

It is necessary to use an internal standard to get high accuracy when HPLC is equipped with MS–MS as the detector. PMPA was adopted because of its similarity of structure, retention time, ionization, and extraction efficiency as well as less endogenous interference in plasma. The structures of adefovir and PMPA are shown in Figure 1.

Separation and selectivity

Negative ESI-MS of adefovir and PMPA are shown in Figure 2,

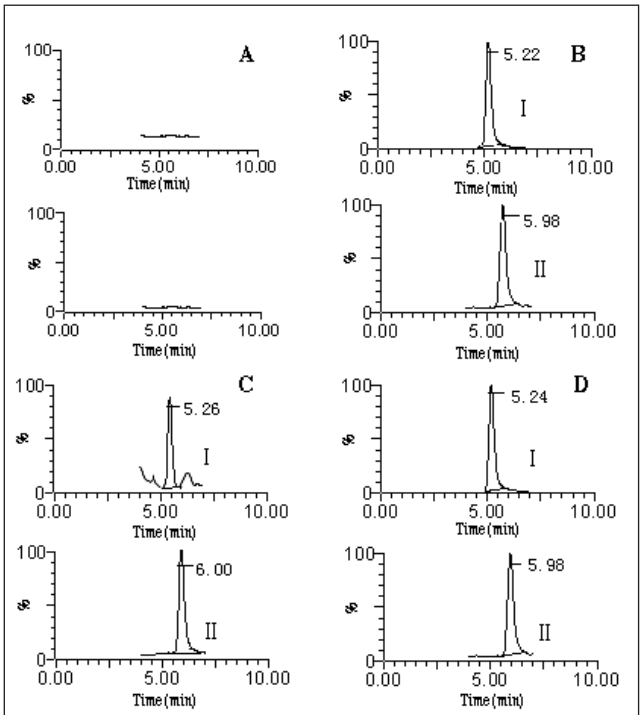

Figure 4. The SRM(–) chromatogram MS–MS of a blank plasma sample, A, supplemented plasma sample (concentration of adefovir = 10 ng/mL), B; LLOQ (concentration of adefovir = 0.20 ng/mL), C; plasma sample obtained at 0.75 h from a subject who received a single oral dose (10 mg), D. The retention times of adefovir and PMPA were 5.2 min and 6.0 min, respectively. Peak I: adefovir; Peak II: PMPA.

respectively. According to the mass spectra, 272.0 *m/z* ([M-H]$^-$) of adefovir and 286. 3 *m/z* ([M-H]$^-$) of PMPA were selected for monitoring. The SRM(–) chromatogram MS–MS of supplemented plasma are depicted in Figure 4B. As shown, the retention times of adefovir and PMPA were 5.2 and 6.0 min, respectively. Short retention times were obtained by using an elution system of methanol–water (20:80, v/v) as the mobile phase. The total HPLC–MS–MS analysis time was 7.0 min per sample. A representative chromatogram of a plasma sample obtained at 0.75 h from a subject who received a single oral dose (10 mg) is shown in Figure 4D.

Method validation

Figure 4A shows an HPLC chromatogram for a blank plasma sample. No significant interference from endogenous substance with analyte or PMPA were detected. The matrix effect on the ionization of analyte or PMPA was evaluated by comparing the peak area of analytes redissolved in extracted blank samples (the final solution of blank plasma after being extracted and redissolved) with that redissolved in the eluent at the same concentration level. All ratios were between 85% and 115%, which meant there was no matrix effect for adefovir and PMPA in this method. The linear regression of the peak ratios versus concentrations was fitted over the concentration range of 0.20–100 ng/mL in human plasma. The mean equation of the standard curve was $R = 0.03200C - 0.001049$ ($r^2 = 0.9962$), where R cor-

Table I. The Intra- and Inter-batch Precision, Accuracy of Adefovir in Human Plasma

Nominal concentration (ng/mL)	Mean concentration found (ng/mL)	Accuracy (%)	Precision (%)	Bias (%)
Intra-batch (*n* = 5)				
0.3	0.32	106.7	9.1	6.7
1.5	1.38	92.0	2.8	–8.0
7.5	7.67	102.3	2.9	2.3
37.5	38.81	103.5	3.3	3.5
Inter-batch (*n* = 5)				
0.3	0.30	98.7	11.3	–1.3
1.5	1.45	96.9	5.1	–3.1
7.5	7.79	103.8	5.0	3.8
37.5	39.49	105.3	4.3	5.3

Table II. Summary of the Stability of Adefovir in Human Plasma (*n* = 5)

Stability conditions	Nominal concentration (ng/mL)	Mean concentration found (ng/mL)	RSD (%)	Bias (%)	Recovery (%)
Long-term (30 days, –40°C)	0.3	0.34	9.6	11.7	111.7
	37.5	36.40	7.4	–2.9	97.1
Short-term (8 h, 20–30°C)	0.3	0.31	9.3	3.0	103.0
	37.5	34.20	7.6	–8.8	91.2
Autosampler (24 h, 8°C)	0.3	0.34	8.2	12.3	112.3
	37.5	38.60	6.3	2.9	102.9
Three freeze-and-thaw cycles (–40°C)	0.3	0.32	9.9	6.7	106.7
	37.5	34.80	5.7	–7.2	92.8
Dried extract (48 h, –40°C)	0.3	0.32	9.1	6.3	106.3
	37.5	37.05	5.3	–1.2	98.8

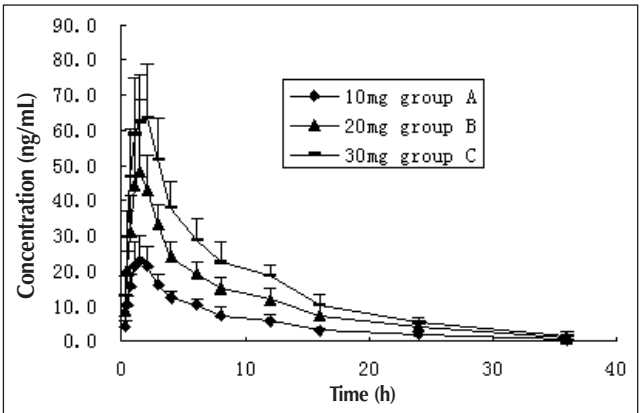

Figure 5. Plasma concentration–time profile of adefovir after an oral administration of 10 mg, 20 mg, and 30 mg adefovir dipivoxil to 36 healthy volunteers (Group A, B, C). Each point represents a mean ± SD (*n* = 12).

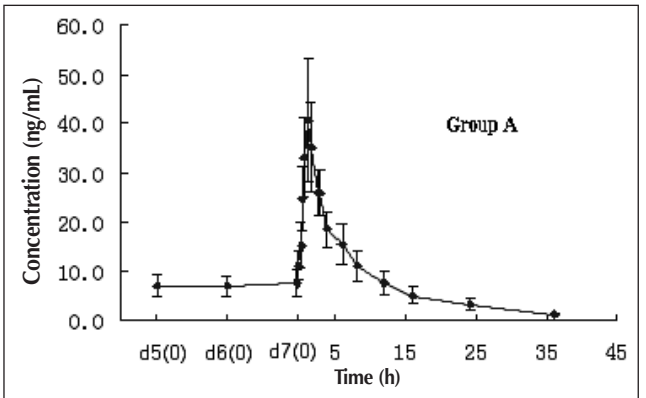

Figure 6. Mean plasma concentration-time curve of adefovir in 12 healthy volunteers (Group A) after oral multidoses of adefovir dipivoxil capsule (10 mg, twice a day). Each point represents a mean ± SD (*n* = 12).

Table III. Mean Pharmacokinetic Parameters for Adefovir After Single Oral Doses of 10 mg, 20 mg, and 30 mg of Adefovir Dipivoxil to Healthy Human Volunteers (*n* = 12)

Parameters (units)	10 mg	20 mg	30 mg
T_{max} (h)	1.5 ± 0.6	1.6 ± 0.7	1.8 ± 0.6
C_{max} (ng/mL)	26.6 ± 6.1	55.7 ± 16.2	70.2 ± 11.8
V/F (L)	512.3 ± 159.4	432.0 ± 116. 9	446.7 ± 124.8
CL/F (L/h)	44.0 ± 12.0	40.2 ± 11.1	41.2 ± 9.39
AUC_{0-36} (ng h/mL)	184.5 ± 25.2	379.3 ± 61.8	556.5 ± 86.7
$t_{1/2}$ (h)	8.0 ± 0.9	7.5 ± 0.8	7.5 ± 0.9
MRT_{0-36} (h)	8.9 ± 0.9	9.0 ± 1.0	8.9 ± 1.0

responds to the peak area ratio of adefovir to the PMPA, and *C* refers to the concentration of adefovir added to plasma over a concentration range of 0.20–100 ng/mL. The LLOQ for adefovir proved to be 0.20 ng/mL. Figure 4C shows the chromatogram of an extracted sample that contained 0.20 ng/mL (LLOQ) of adefovir.

Data for intra-batch and inter-batch precision and accuracy of the method for adefovir are presented in Table I. The accuracy deviation values are within 8.00% for 0.3, 1.5, 7.5, and 37.5 ng/mL of the actual values. The precision determined at each concentration level does not exceed 11.30% of the relative standard deviation (RSD). The mean extraction recovery of adefovir, determined at four concentrations (0.3, 1.5, 7.5 and 37.5 ng/mL), were 70.4, 77.6, 85.5, and 79.4% (n = 5), respectively. The extraction recovery determined for adefovir was shown to be consistent, precise, and reproducible. Table II summarizes long-term stability, short-term stability, autosampler stability, dried extract stability, and the freeze-and-thaw stability data of adefovir, which showed the stability of the samples.

Results of pharmacokinetic study

The developed method was successfully used for a pharmacokinetic study in which plasma concentration of adefovir in 36 healthy Chinese volunteers were determined up to 36 h after the oral administration of 10 mg, 20 mg, and 30 mg adefovir dipivoxil capsules. Plasma drug concentration-time curves are shown in Figure 5; plasma drug concentration-time curves of multidoses are shown in Figure 6.

The mean pharmacokinetic parameters of single doses for the adefovir dipivoxil capsule are presented in Table III. The main pharmacokinetic parameters of multidoses were as follow: Css_{max}, (45.0 ± 9.1) ng/mL; t_{max}, (1.4 ± 0.4) h; Css_{min}, (7.3 ± 2.3) ng/mL; C_{av}, (17.0 ± 2.7) ng/mL; *DF*, (2.2 ± 0.5); *AUCss*, (204.5 ± 32.7) ng h/mL.

Conclusion

The described LC–ESI-MS–MS method for determination of adefovir in human plasma was proven to be rapid, selective, and sensitive. These make the method suitable for the analysis of a large number of samples resulting from the pharmacokinetic, bioavailability, or bioequivalence study of adefovir in humans. There were no adverse events during the conduct of the study. The pharmacokinetic parameters adefovir can provide some information for clinical medication.

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