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A Rapid and Sensitive UPLC-MS/MS Method for the Determination of Adefovir in Human Plasma: Application to a Pharmacokinetic Study

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Abstract

A sensitive, rapid and selective liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) analytical method based on protein precipitation was developed and validated for analysis of Adefovir in human plasma. Adefovir-d4 was used as an internal standard and Waters X-Select HSS T3-C18 (3.0 × 50 mm, 2.5 µm) column provided the desired chromatographic separation of compounds followed by detection with mass spectrometry. The method used simple isocratic chromatographic condition and mass spectrometric detection in the positive ionization mode. The calibration curves were linear over the range of 1.00 ng/mL to 30.00 ng/mL with the lower limit of quantitation validated at 1.00 ng/mL. The degree of matrix effect for Adefovir was determined in six different sources of human plasma was 5.23% and had no impact on study samples analysis with the shortest runtime obtained (1.5 min). The intra- and inter-day precision values were within 2.37% and 7.87%, respectively, for Adefovir at the lower limit of quantification level. The method described here was successfully applied for the evaluation of pharmacokinetic profiles of Adefovir after single oral administration doses of 10 mg Adefovir dipivoxil to 28 healthy volunteers.

Keywords: Adefovir; Liquid chromatography; Tandem mass spectroscopy; UPLC-MS/MS; Pharmacokinetic study; Bioequivalence study

Introduction

Adefovir, an acyclic phosphonate analog of deoxynucleoside mono-phosphate (IUPAC name: {[2-(6-amino-9H-purin-9-yl) ethoxy] methyl} phosphonic acid, PMEA), is a broad spectrum antiviral agent acting as a DNA polymerase inhibitor [1]. It has activity against herpes virus (Epstein-Barr) and retroviruses including the human immunodeficiency virus (HIV) [1]. Adefovir is largely used to treat chronic hepatitis B in adults, though the drug is reported for poor oral bioavailability [2]. The oral bioavailability of Adefovir has been substantially improved by using the bis-pivaloyloxymethyl ester of Adefovir (bis-POM PMEA, Adefovir dipivoxil (Figure 1) as a pro-drug with enhanced lipophilicity and achieving higher systemic Adefovir levels. Adefovir dipivoxil spontaneously hydrolyzes to mono-POM-PMEA, which is rapidly converted into PMEA (Adefovir) by enzyme. Adefovir is an acyclic nucleoside analog of adenosine monophosphate which is phosphorylated to the active metabolite Adefovir diphosphate by cellular kinases [2].

Although several methods have been reported to quantify Adefovir in human plasma [3-6] including serum [7], by employing liquid chromatography-tandem mass spectrometry (LC-MS/MS), analytical limitations could not be overcome. The published methods demonstrated LC-MS/MS method for Adefovir estimation but lacked sensitivity and had lengthy runtime [5,7]. Xie et al. [6] developed a LC-MS/MS method for the determination of Adefovir with limit of quantitation 0.5 ng/mL but this method had matrix related issue.

The reported method failed to use labeled/deuterated analog of Adefovir for estimation from plasma to compensate equivalent matrix effect with that of analyte. Vela et al. [8] had developed an LC-MS/MS method using a very tedious and complexion-pairing technique for Adefovir estimation. An interesting LC-MS/MS method of Adefovir had been reported with emphasis on hydrophilic interaction but it failed to achieve lower limit of quantification (LOQ) 1.00 ng/mL [9]. Moreover, Chen et al. [10] and Goswami et al. [11] achieved sensitivity 0.25 ng/mL and 0.5 ng/mL, respectively, But the methods had lengthy analysis runtime (>7 min) and (4.5 min), respectively (Figure 1).

Bioavailability/bioequivalence studies are frequently conducted on healthy volunteers with Adefovir dipivoxil 10 or 20 mg tablet, marketed as Hespera (Gilead Sciences, Inc., Foster City, CA). Regulatory guidance [12,13] suggests that LOQ should be sufficient to characterize pharmacokinetic parameters based on expected peak plasma concentration (Cmax). European Medicine Agency [13] suggests 5% of Cmax should be achieved to have sufficient sensitivity to capture profile in elimination phase of a drug. Amonograph on Adefovir states that the following oral administration of a 10 mg single dose of Hespera in

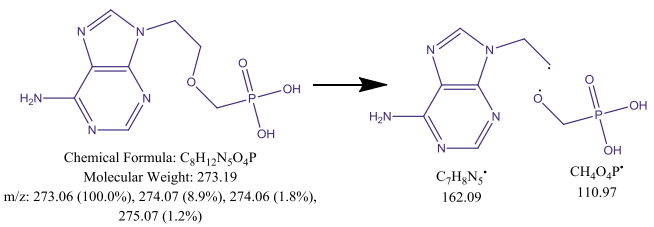

Figure 1: Chemical Structure of Adefovir and its fragments.

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chronic hepatitis B patients, the mean Cmax was 18.4 ng/mL with mean elimination half-life of 7.48 h [14]. But, published literature reflected high variation (14.9 ng/mL to 29.7 ng/mL) in mean Cmax for 10 mg Adefovir tablet, though administered to healthy volunteers [5,10]. Such variation could be attributed to matrix effect or any other aspects of method limitations. Therefore, it becomes imperative to develop a precise, accurate, and high throughput method for estimation of Adefovir in human plasma. For conducting the bioequivalence study on Adefovir (i.e. 10 mg Hespera tablet), method sensitivity should be such that concentration profile up to 36 h (5 half-lives) could be plotted. Though 1.0 ng/mL LOQ could have sufficed [13] to characterize pharmacokinetic parameter, we further decreased method sensitivity to this concentration. In the present study, a systematic evaluation of matrix interference was investigated by using protein precipitation extraction (PPE) followed by a cleaning step. The combination technique was used to bring down matrix effect below 6% level effectively. The unique method highlights Adefovir stability as well as selectivity in blank (untreated) plasma, hemolyzed and lipemic plasma samples. The method had been successfully applied to clinical sample analysis.

Experimental

Chemicals and reagents

Working standards of Adefovir (Purity=98.0%) and Adefovir-d_4 (deuterium labeled Adefovir, purity=98.0%) were procured from Hetero Corporate, Telangana, India, and Toronto Research Chemicals, Canada, respectively. Acetic acid, ammonium acetate and ammonia solution were Fisher (Analysis grade), methanol is Fisher (gradient grade). Acetonitrile and Dichloromethane were Sigma (gradient grade). De-Ionized ultrapure water was obtained by using a Milli-Q device (Millipore, Cairo, Egypt). Drug-free (blank) human plasma containing K3EDTA (ethylene-diaminetetraacetic acid tripotassium salt), as anticoagulant, was obtained from the subjects who have participated in clinical studies.

Preparation of standards and quality control samples

The stock solutions of Adefovir were prepared by dissolving the accurately weighed reference compound in methanol to give a final concentration of 100.00 µg/mL. Two separate stock solutions of Adefovir were prepared for bulk spiking of calibration standards and quality control (QC) samples. Primary dilutions and working standard solutions were prepared from stock solutions using methanol to obtain standard working solutions at concentrations of 0.1, 0.2, 0.4, 0.6, 1.0, 1.6, 2.2 and 3.0 µg/mL, which used for spiking the calibration standards. However, concentrations 0.3, 1.2 and 2.4 µg/mL were used for spiking QC samples. The stock solution of the internal standards (IS) was prepared by dissolving the accurately weighed the standard of Adefovir-d_4 in methanol to give a final concentration of 96.0 µg/mL, then a diluted solution was prepared of concentration 96.0 ng/mL. All the solutions were stored at 4°C and were brought to room temperature before use. Eight-point calibration standards and QC samples were prepared by spiking the blank human plasma with appropriate concentration of Adefovir. Calibration standards samples were prepared at concentrations of 1.0, 2.0, 4.0, 6.0, 10.0, 16.0, 22.0 and 30.0 ng/mL. QC samples (QC_L, QC_M and QC_H) were prepared at concentrations of 3.0, 12.0 and 24.0 ng/mL, respectively. The spiked Calibration standards and QC samples were stored at -80°C.

Plasma sample preparation

250 mL of plasma samples were pipetted into disposable Eppendorf tubes and 50 µL of IS working solution (96.0 ng/mL of IS) was added. Vortex for 30 s and leave samples for 5.0 min. Samples were pretreated with 200 µL of 10% acetic acid and vortex the samples for 30 s. Precipitate with 500 µL of acetonitrile then vortex for 30 s, and centrifuge the samples for 10 min at 4000 rpm. Decant 700 µL of supernatant in new labeled Eppendorf tubes. Add 700 µL of dichloromethane then vortex for 30 s and centrifuge samples for 10 min at 4000 rpm then decant from upper layer (aqueous) 200 µL in new labeled tubes. Evaporate the samples in concentrator for 10 min at 60°C to get rid of traces dichloromethane. Transfer samples to auto-sampler racks and inject 10 µL.

UPLC-MS/MS instrumentation and analytical conditions

Liquid chromatography was performed on an Acquity H-Class ultra-performance liquid chromatography (UPLC) unit (Waters Corp., USA) comprising Acquity UPLC Pump with Quaternary solvent manager, a cooling auto-sampler, a column oven of temperature control. Chromatographic separations were achieved on Waters X-Select HSS T3-C18 (3.0 × 50 mm, 2.5 µm) column using a mobile phase mixture of 0.025 M ammonium acetate buffer and 2% ammonia in methanol (90:10, v/v), at isocratic flow rate of 0.5 mL/min. The column and auto-sampler temperature were kept at 40°C and 8°C, respectively. XEVO TQ-MS/MS triple quadruple mass spectrometer equipped with an electro-spray ionization (ESI) source, which was operated in positive ion mode. Quantification was carried out using multiple reactions monitoring (MRM) mode of the transitions m/z 274.04>162.09 and 278.04>166.09 for Adefovir and Adefovir-d4, respectively. Dwell time was set at 36 ms for Adefovir and IS. Nitrogen was used as the nebulizer, auxiliary, collision and curtain gas. The source parameters of the mass spectrometer were optimized and maintained as follows: Capillary: 1.5 KV, cone: 35 V, collision energy: 28 eV, source temp.: 150°C, desolvation gas: 800 L/H, desolvation temp.: 300°C, collision gas flow: 0.15 mL/min (Figure 2).

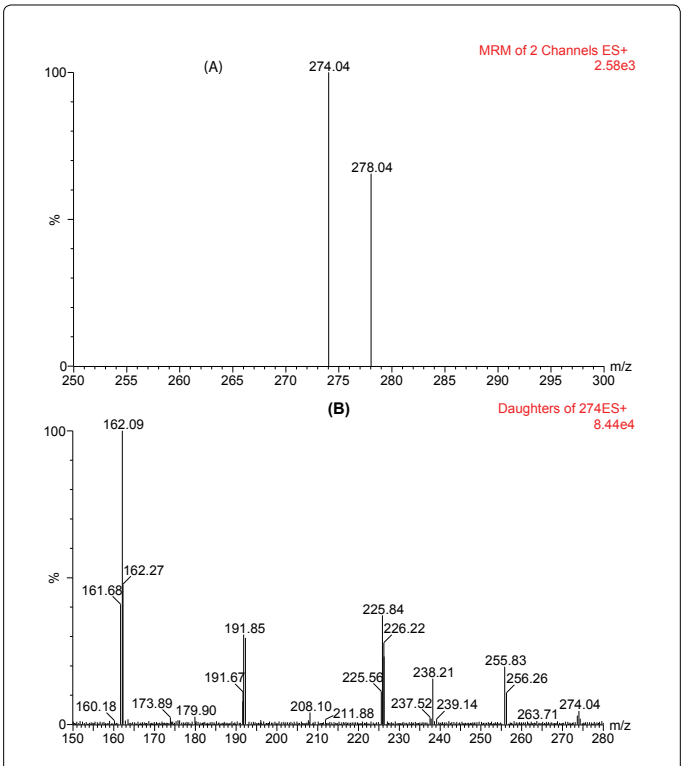

Figure 2: (A) Mass spectrum for Adefovir and Adefovid-d_4 in the positive ESI mode; **(B)** MS/MS product ion spectrum of $[M+H]^+$ of Adefovir at m/z 274.04 as the precursor ion.

Method validation

Method validation of Adefovir in human plasma was carried out, following US Food and Drug Administration guidelines and Guidance from European Medicine Agency [12,13]. The method was validated for selectivity, sensitivity and carry over, linearity, precision and accuracy, recovery, matrix effect, re-injection reproducibility, dilution integrity and stability of Adefovir during both short-term sample processing and long-term storage.

Selectivity: The selectivity of the method towards endogenous plasma matrix components, and concomitant medications was assessed in six different sources of blank plasma samples; these were collected under controlled conditions to simulate the plasma matrix harvested from participants. These samples were processed using the proposed extraction protocol and analyzed with the set chromatographic conditions at LLOQ level. The peak area of the co-eluting components or interferences in blank sample should be less than 20% and 5% from those of the analyte and IS, respectively (Figure 3).

Sensitivity: The procedures adopted for investigating method sensitivity differentiated between two types of sensitivities namely: (A) calibration sensitivity, which was equated with the slope of the calibration graph, and (B) analytical sensitivities (γ), which accounted for the variation in the standard deviation of the analytical signal measured for different concentration levels of Adefovir.

Linearity and LLOQ: Six calibration curves were used to demonstrate the linearity of the method. The area ratio responses for (Adefovir/IS) was used for regression analysis. Each calibration curve

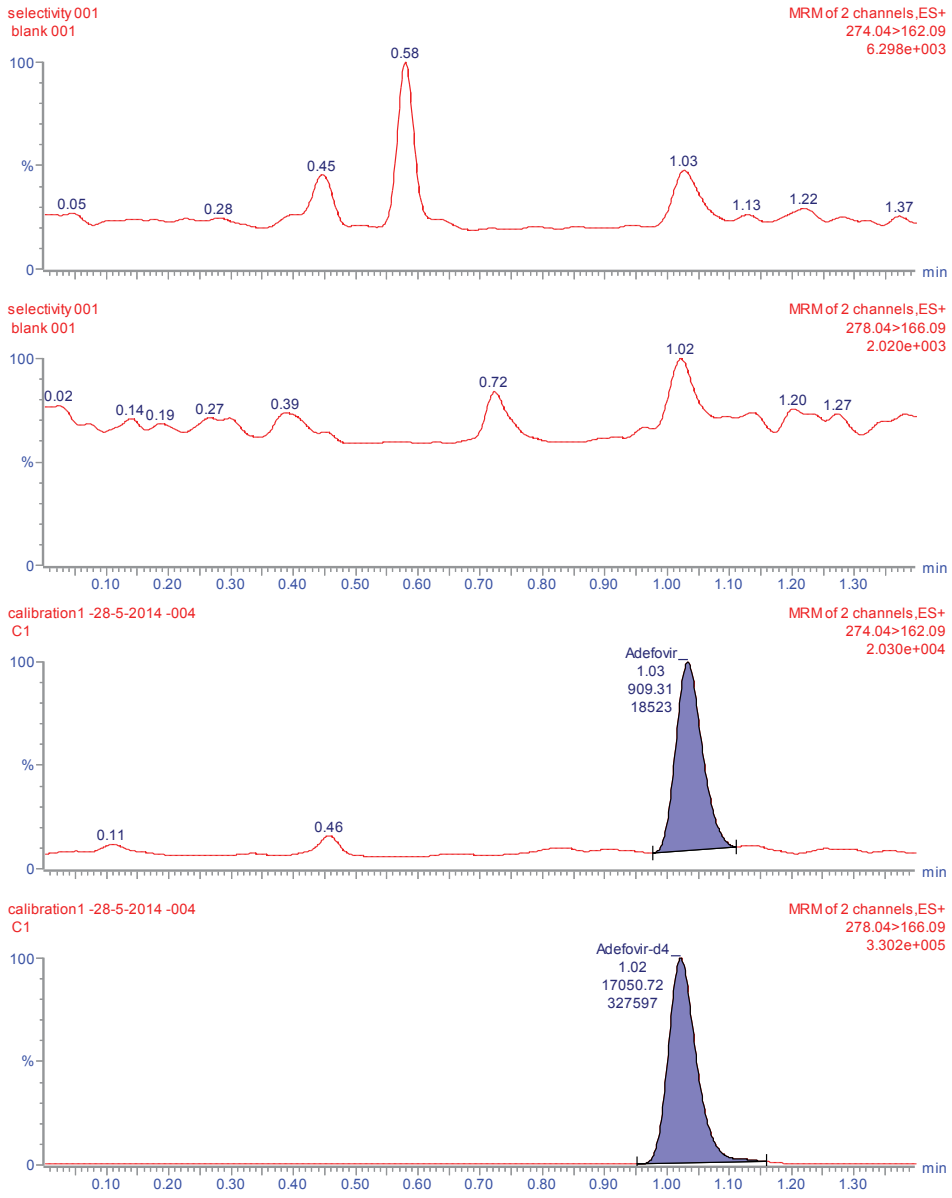

Figure 3: Chromatograms of (a) Drug-free human plasma; (b) Spiked plasma at lower limit of quantitation.

was analyzed individually by using least square weighted ($1/x^2$) linear regression (obtained by best fit method). Back-calculations were made from these curves to determine the concentration of Adefovir in each calibrator. A correlation coefficient (r) >0.99 was desirable for all the calibration curves. In addition, the analyte peak at LLOQ concentration should be identifiable, discrete and reproducible with accuracy within ± 20% and a precision ≤ 20% whereas other levels of concentration should be ± 15%for accuracy and ≤ 15 for precision (Table 1).

Precision and accuracy: The intra- and inter-day precision and accuracy were performed for Adefovir in human plasma, which determined by replicate analysis of QC samples (n=6) at LLOQ, QCL, QCM and QCH. The precision of the method was determined by calculating the percentage coefficient of variation (%CV) for each level. The deviation at each concentration level from the nominal concentration was expected to be (≤ 15%), excluding at LLOQ level (≤ 20%). Similarly, the mean accuracy should not deviate by (± 15.0%), excluding at LLOQ level (± 20%) (Table 2).

Matrix effect: The matrix effect was estimated by extraction of 6 lots of blank matrix from individual donors. The matrix factor (MF) was estimated by calculating the ratio of the peak area in the presence of matrix (measured by analysing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (pure solution of the analyte). The IS normalized MF should also be calculated by dividing the MF of the analyte by the MF of the IS. When, IN-NMF=1 indicates no matrix effect, IN-NMF<1 indicates ion-suppression and IN-NMF>1 indicates ion-enhancement. The CV of the IS-normalized MF calculated from the 6 lots of matrix should not be greater than 15% (Table 3).

Stability: Primary stock solutions were found to be stable for 151 days. Stock solution stability was studied at two concentration levels and it was found to be 99.23% to 107.84%.

Results of short-term stability are shown in Table 4. Samples were stable under the studied conditions. Three sets of spiked samples with low and high concentrations of the analytes were analyzed and left in the auto-sampler at 8°C for one day. The samples were analyzed using a freshly prepared calibration samples. The processed samples were stable at room temperature for this period. The results are shown in Table 4.

The long-term stability of frozen plasma samples was examined after 151 days storage at -86°C. The samples were stable under studied conditions as shown in Table 4.

Plasma samples with low, medium and high concentrations of Adefovir were prepared. The samples were stored at -86°C and subjected for 3 freeze/thaw cycles. During each cycle triplicate one ml aliquots was processed, analyzed and the results averaged. No substance loss during repeated thawing and freezing was observed as shown in Table 4.

Calibrators and QCs	Adefovir concentrations (ng/mL)
Blank	-
C1	1
C2	2
C3	4
C4	6
C5	10
C6	16
C7	22
C8	30
QC_L	3
QC_M	12
QC_H	24

Table 1: Concentrations of matrix based calibrators and QCs Samples.

Precision between the three runs				
Result	**LLOQ**	**QC_L**	**QC_M**	**QC_H**
Average	1.055	2.8	11.994	25.463
ST.DEV	0.083	0.142	0.626	0.759
CV%	7.87	5.07	5.22	2.98
Accuracy between the three runs				
Result	**LLOQ**	**QC_L**	**QC_M**	**QC_H**
Average	1.055	2.8	11.994	25.463
Error	0.055	-0.2	-0.006	1.463
Relative error (%)	5.5	-6.67	-0.05	6.1

Table 2: Between run accuracy and precion.

Pharmacokinetic Study

The validated UPLC-MS/MS method was applied to a PK study of Adefovir Dipivoxil (10 mg film-coated tablet), Twenty-Eight Egyptian healthy adult volunteers participated in this study. The volunteers were all adult Egyptians who were selected after completing a thorough medical, biochemical and physical examination. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved by the Human Investigation Ethical Committee at the Ministry of Health, Egypt.

After an overnight fast (12 h), the volunteers took the assigned

IS-Normalized MF		
Source	**QC_L**	**QC_H**
Source (1)	1.2022	1.0508
Source (2)	1.2177	1.1676
Source (3)	1.1229	1.1331
Source (4)	1.1446	1.1552
Source (5)	1.1897	1.0825
Source (6)	1.1796	1.0277
Average IS-N MF	1.1761	1.1028
ST.DEV IS-N MF	0.0359	0.0577
CV%	3.05	5.23

Table 3: Matrix effect.

Parameters	**Stability%**		
a) Post preparative (Auto-sampler) stability	0 h (RT)		24 h (RT)
QC_L	92.23		90
QC_H	105.86		106.93
b) Matrix based short term stability	0 h (RT)		24 h (RT)
QC_L	89.93		86.9
QC_H	104.65		103.84
c) Matrix based long term stability	0 day	41 days	151 days
QC_L	94.7	94.13	93.13
QC_H	96.63	99.66	95.54
d) The freeze and thaw stability	Cycle (0)		Cycle (3)
QC_L	93.83		93.23
QC_H	105.24		109.36

Table 4: Stability of Adefovir in human plasma by the proposed method.

tablet orally with 250 mL of water. The volunteers were treated with an oral dose of 10 mg of Adefovir Dipivoxil, and with 10 mg after a 1-week washout period. Regular standardized low-fat meals were provided until 4 h after dose administration; water intake was allowed after 2 h. Following drug administration, venous blood samples (5 mL) were collected into heparinized tubes according to the following schedule: immediately before administration and 0.25 h, 0.5 h, 0.75 h, 1 h, 1.25 h, 1.5 h, 1.75 h, 2h, 2.25 h, 2.5 h, 2.75 h, 3 h, 3.5 h, 4 h, 5 h, 6 h, 8 h, 10 h, 12 h, 24 h, 36 h and 48 h after dosing.

Blood samples were centrifuged at 4000 rpm for 10 min to obtain the plasma. The plasma samples were labeled and kept frozen at 86°C until analysis. The plasma concentration of Adefovir vs. time profiles were obtained for each individual subject, and non-compartmental PK parameter calculations were performed using the WinNonLin Program version 2.0. The area under the plasma Adefovir concentration-time curve (AUC) was calculated using the trapezoidal rule extrapolated to infinite time. Cmax was the observed maximum concentration and Tmax was the time to reach the maximum drug concentration.

Bioequivalence Discussion

Figure 4 shows the mean plasma concentrations of Adefovir, while Table 5 shows the pharmacokinetic parameters of Adefovir following oral administration of one tablet of DOVOCARE 10 mg (test product), and one tablet of HEPSERA® 10 mg (reference product). The point estimate of the test formulation to the reference formulation ratios (T/R) for AUC_{0-48} was 97.54 with 90% confidence limits (94.47→ 100.72). For AUC_{0-inf}, it was 98.68 with 90% confidence limits (95.26→ 102.23) and for C_{max}, it was 99.22 with 90% confidence intervals (94.78→ 103.86). The parametric 90% confidence intervals of the mean values for the test/reference ratio were, in each case, within the bioequivalence acceptable boundaries of 80.00% to 125.00% for the pharmacokinetic parameters AUC_{0-48}, AUC_{0-inf} and C_{max}. And the calculated power of the study was 100%. The parametric 90% confidence intervals of the mean values for the test/reference ratio were, in each case, within the bioequivalence acceptable boundaries of 80.00% to 125.00% for the pharmacokinetic parameters AUC_{0-48}, AUC_{0-inf} and C_{max}. The difference between means of T_{max} is not significant (P>0.05) with respect to Kruskal Wallis test [15,16].

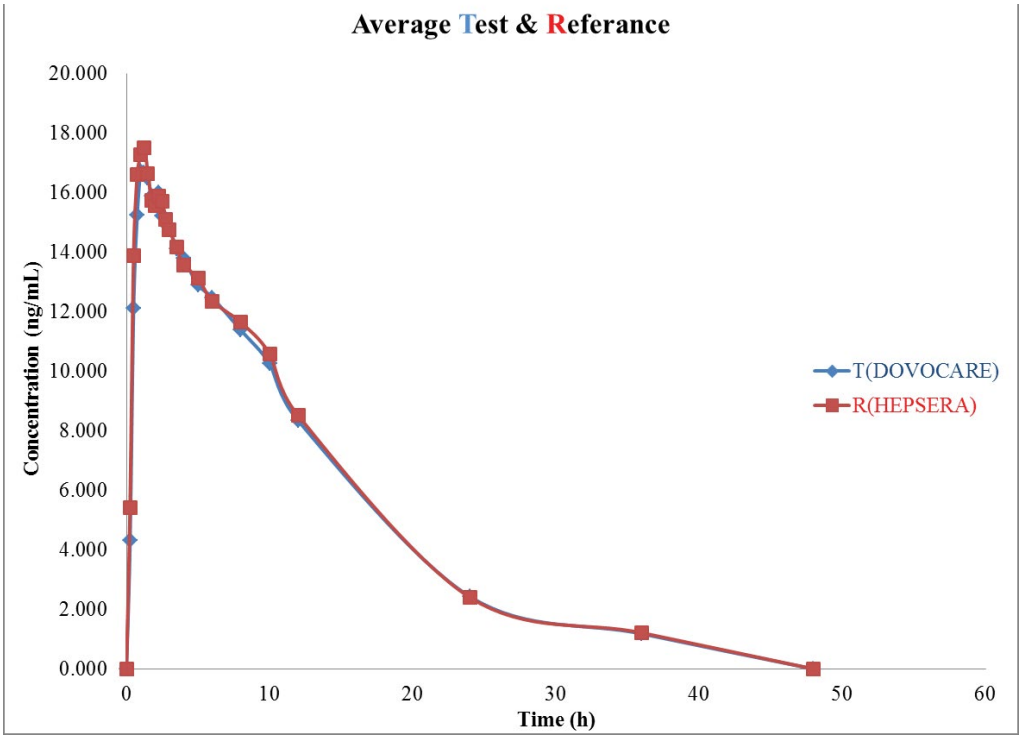

Figure 4: Average plasma concentration (ng/ml) of Adefovir vs. time (hours) curves following a single dose of 10 mg Adefovir tablet (T: DOVOCARE and R: HEPSERA) based on 28 participants.

Parameter	Point estimate (ratio of geometric mean %)	Confidence interval		Confidence level	Standard bioequivalence limit		Conclusion
		Lower limit%	Upper limit%	(1-2a)%	Lower limit%	Upper limit%	
$AUC_{0@last}$	97.544	94.471	100.718	90	80	125	BE
AUC_{0-inf}	98.68	95.257	102.227	90	80	125	BE
C_{max}	99.215	94.778	103.859	90	80	125	BE

Table 5: Confidence interval for the ratio of geometric mean (μT/μR).

The results of this bioequivalence study showed the equivalence of the two studied products in terms of the rate of absorption as indicated by T_{max} and C_{max} and in terms of the extent of absorption as indicated by AUC_{0-48} and AUC_{0-inf} (Figure 4 and Table 5).

Conclusion

In summary, a rapid, specific, reproducible, high-throughput and sensitive UPLC-MS/MS method allows determination of Adefovir using Adefovir-d4 as an internal standard was developed and validated.

Overall the developed method presented adequate sensitivity, excellent selectivity and desired reproducibility for the quantification of Adefovir in human plasma. The other major advantage of this validated method is the shorter runtime of 1.5 min, allowing the quantitation of over 500 samples per day. Moreover, the low limit of quantitation (1 ng/ml), the low matrix effect, the precision and accuracy of the method and also the stability are well within the limits required for bioanalytical assays. In addition, the ISR result with 96.64% at the end of the study further added strength to our current method.

All these advantages would make it efficient for routine therapeutic drug monitoring as well as for the analysis of large number of plasma samples obtained from exploratory pharmacokinetic studies.

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