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Journal of Chromatography B, 852 (2007) 382–388
JOURNAL OF CHROMATOGRAPHY B
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Development and validation of a high-performance liquid chromatography–tandem mass spectrometry for the determination of penciclovir in human plasma: Application to a bioequivalence study

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Received 10 October 2006; accepted 28 January 2007
Available online 3 February 2007

Abstract

We developed and validated a simple high-performance liquid chromatography (HPLC) coupled with positive ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) detection system for determining penciclovir (active metabolite of famciclovir) levels in human plasma using acyclovir as an internal standard (IS). Acquisition was performed in multiple reaction monitoring (MRM) mode by monitoring the transitions: *m/z* 254.00 > 152.09 for penciclovir and *m/z* 226.00 > 152.09 for IS. The analytes were chromatographed on a Capcellpak MGII C_{18} reversed-phase chromatographic column by isocratic elution using 30% methanol and 70% Milli-Q water containing 10 mM ammonium formate (adjusted to pH 3.1 with formic acid). Results were linear over the studied range (0.05–10 μg/ml) with $r^2 = 0.9999$, and the total analysis time for each run was 2 min. Intra- and inter-assay precisions were 2.3–7.8 and 3.7–7.5%, respectively, and intra- and inter-assay relative errors (RE) were 2.0–8.4 and 1.9–9.1%, respectively. The lower limit of quantification (LLOQ) was 0.05 μg/ml. At this concentration mean intra- and inter-assay precisions were 7.8 and 7.5%, respectively, and mean intra- and inter-assay relative errors were 2.2 and 9.1%, respectively. Penciclovir was found to be stable in plasma samples under short-, long-term storage and processing conditions. The developed assay was successfully applied to a bioequivalence study of penciclovir administered as a single oral dose (500 mg as famciclovir) to healthy volunteers.

Keywords: Penciclovir; Famciclovir; Acyclovir; LC–MS/MS; Human plasma; Bioequivalence study

1. Introduction

Herpesvirus infection is inhibited by the antiviral agent, penciclovir (2-amino-9-[4-hydroxy-3-(hydroxymethyl)butyl]-3H-purin-6-one), both in vitro and in vivo [1,2]. Moreover, penciclovir is a potent and highly selective inhibitor of herpes viruses, such as, herpes simplex virus type 1 and 2, varicella-zoster virus, Epstein virus, and of hepatitis B virus [3–5]. However, penciclovir has poor bioavailability when administered orally to human [6]. To improve this poor oral bioavailability, the diacetate ester of the 6-deoxy derivative of penciclovir, famciclovir ([2-(acetyloxymethyl)-4-(2-aminopurin-9-yl)butyl]acetate), was developed as a prodrug of penciclovir [7–9]. After orally administering famciclovir to rats and dogs, urinary recoveries of penciclovir were found to represent 36.0 and 36.4% of the administered doses, respectively [10]. In humans, more than half of an oral dose of famciclovir was found to be absorbed and converted rapidly to penciclovir [6].

Penciclovir, like acyclovir and ganciclovir, is a nucleoside analogue that shares structural similarity with endogenous compounds. Therefore, selective analytical methods are required to analyze its levels and those of its analogous in biological fluids [11]. In previous pharmacokinetic studies [10,12–14], penciclovir was analyzed by high-performance liquid chromatography (HPLC) with UV [15–17] or fluorescence detection [18]. However, these methods suffer from a number of disadvantages, including inadequate sensitivity (a lower limit of quantification (LLOQ) up to 0.1 μg/ml) and require extensive

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doi:10.1016/j.jchromb.2007.01.044

sample preparation and large biological volumes (i.e., up to 0.5 ml of plasma).

Nowadays, liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) has become a popular analytical technique for determining drugs levels in biological fluids, since it can provide higher sensitivity and selectivity than other traditional methods. To our knowledge, no report has been issued concerning the quantitative analysis of penciclovir using tandem mass spectrometry. The objective of the current study was to develop a rapid, selective, and sensitive liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method for the analysis of penciclovir in human plasma. In order to test the applicability of the devised method, we used it to perform a bioequivalence study in 24 healthy male Korean volunteers administered famciclovir in tablet form (500 mg).

2. Experimental

2.1. Materials and reagents

Penciclovir and acyclovir (internal standard, IS) were obtained from TEVA-ASSIA Chem. Co., Ltd. (Beer Sheva, Israel) and Sigma–Aldrich (St. Louis, MO, USA), respectively (Fig. 1). Solvents were of HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA), and a Milli-Q® (Millipore Co., Milford, MA, USA) water purification system was used to obtain purified water for HPLC. All other chemicals and solvent were of the highest analytical grades available.

2.2. Calibration standard and quality control samples

A stock solution of penciclovir was prepared by dissolving penciclovir in dimethyl sulfoxide (DMSO) to 1 mg/ml. This stock solution was appropriately diluted with purified water to obtain working standards for calibration purposes. Calibration curves of penciclover were prepared by spiking blank plasma at 0.05, 0.1, 0.5, 1, 5 and 10 μg/ml. Quality control (QC) samples (0.05, 0.5 and 10 μg/ml) were also prepared by diluting stock solution. In order to prepare stock solutions (1 mg/ml) of IS, 10 mg of acyclovir was dissolved in 10 ml of DMSO. This solution was further diluted with purified water to a final concentration of 200 μg/ml. All solutions were stored at −80 °C.

2.3. Instrumentation and chromatographic conditions

A Waters 2795 HPLC system and a Waters Micromass Quattro Premier triple quadrupole mass spectrometer equipped with a turbo electrospray interface in positive ionization mode (Waters Ltd., Watford, UK) were used for the LC–MS/MS analysis. Two channels of positive ion multiple reaction monitoring (MRM) mode were used to detect penciclovir and IS. The most abundant product ions of compounds were at *m/z* 152.09 from the parent *m/z* 254.00 ion of penciclovir and at *m/z* 152.09 from the *m/z* 226.00 of IS. Data acquisition was performed using Micromass Masslynx 4.0 and data processing conducted using a Quanlynx data analysis program.

The analytical column used was a Capcellpak MGII C_{18} (50 mm × 2.0 mm i.d. 3 μm, 12 nm; Shiseido, Kyoto, Japan), and the mobile phase consisted of 30% methanol and 70% Milli-Q water containing 10 mM ammonium formate (adjusted to pH 3.1 with formic acid), and was filtered and degassed before use. A flow rate of 0.2 ml/min was used for sample analysis. The temperatures of the autosampler and column oven were 4 and 40 °C, respectively.

2.4. Sample preparation

Plasma samples were stored at −80 °C and allowed to thaw at room temperature before processing. Briefly, a plasma sample (250 μl) was placed in a 1.7 ml Eppendorf tube, and after adding 20 μl of a 200 μg/ml solution of IS, the tube was briefly vortexed and 1 ml of cold methanol added. After vortexing for another 10 min, the tube was centrifuged at 10,000 rpm for 10 min at 4 °C, and 10 μl of the supernatant was then injected onto the analytical column.

2.5. Validation

The strategy applied to validate the devised method was based on the approach detailed in the KFDA guidelines [19]. This procedure allowed us to confirm the linearity over the tested penciclovir concentration range, and to determine the precision, accuracy, and selectivity of the devised method. Selectivity was determined by analyzing plasma samples from six different sources. Precision and accuracy of the method were estimated using replicated samples ($n = 5$). Intra-day precisions were evaluated by analyzing QC samples five times over 1 day, whereas inter-day precisions were evaluated by analyzing QC samples on 5 different days. Variance of precision was calculated from estimated concentrations, and precision is expressed as the relative standard deviation (R.S.D.) at each level. The accuracy of the assay was defined as relative error (RE).

A calibration curve was constructed by plotting peak area ratios of penciclovir to IS against penciclovir concentrations. Unknown sample concentrations were calculated using the regression equation of the calibration curve. Limit of detection

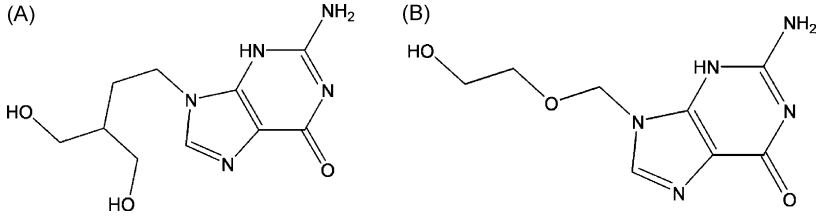

Fig. 1. Structures of: (A) penciclovir and (B) IS.

(LOD) was defined as three times the signal to noise ratio (S/N) and lower limit of quantification was defined as the lowest concentration for which the method is validated with precision and accuracy that fall within the ranges recommended by the KFDA.

2.6. Matrix effect study

This study was conducted to assess the interference caused by plasma matrix during analysis. Three replicates of blank plasmas were processed by cold methanol and the supernatants so obtained were transferred to clean microtube. Then, the supernatants were spiked with working standards (final concentrations were 0.05, 0.5, and 10 μg/ml) and IS (20 μl of a 200 μg/ml). Another three replicates of reference standards were prepared by spiking with the same concentration of penciclovir and IS in methanol. The percentage of matrix effect was calculated as follows:

$$\text{Percentage of matrix effect} = \frac{b}{a} \times 100\%$$

where a and b are the peak area ratios of the penciclovir to IS in solution of pure compounds and plasma extract, respectively.

To assess 'lot-to-lot' matrix variation, five different lots of blank plasma were used to prepare samples at three concentrations (0.05, 0.5, and 10 μg/ml). The relative standard deviations of the peak area ratios of each penciclovir versus IS among the five lots were calculated to determine inter-lot matrix variability.

2.7. Stability

The stability of penciclovir was assessed by analyzing QC samples exposed to different temperatures for different times. QC samples were prepared in sufficient volume to allow multiple replicates ($n = 5$) for each test condition. Results were compared with those of freshly prepared QC samples, and percentage concentration deviations were calculated. The precision is expressed as the relative standard deviation at each level and the accuracy of the assay was defined as relative error. The protocol for the stability study included short- and long-term stability. The short-term stability included: (a) freeze–thaw stability, which was determined after three freeze–thaw cycles on consecutive days; (b) exposure of samples to room temperature for 6 h; (c) exposure of samples to room temperature for 1 and 2 days; (d) exposure to 4 °C (refrigerator) for 1 day; (e) exposure to −80 °C (deep freezer) for 1 day; (f) dilution of the samples two and four times, using blank plasma; (g) the stability of penciclovir in mobile phase at room temperature for 12 h. Long-term stability included: (a) analysis of samples, which were frozen and thawed after 30 days at −80 °C; (b) stability of penciclovir in mobile phase, which were kept upon refrigeration (4 °C) for 20 days.

2.8. Pharmacokinetic study

The study population consisted of 24 healthy male Korean volunteers with an average age of 23.5 years and an average weight of 69.5 kg. Each volunteer was administered a single 500 mg famciclovir orally using a standard 2 × 2 cross-over model in a randomized order (Famvir tablet [Novartis Korea. Ltd., Korea] and Kukje famciclovir tablet [Kukje pharm. Co., Ltd., Korea]). A 1-week washout period was allowed between doses. Approximately 7 ml blood samples were collected via the cannula at the following times; predose, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h after administration. Blood samples were centrifuged immediately, and plasma samples were frozen at −80 °C until required for LC–MS/MS analysis. This study was approved by the Korean Food and Drug Administration (KFDA) and Kyung-Hee University Ethical Committee before obtaining written consents from all volunteers.

Finally, C_{max} values (maximum plasma concentrations) and T_{max} times (times to C_{max}) were determined using individual plasma concentration–time profiles. A non-compartmental model for extravascular input, provided by WinNonlin Professional 3.1 software, was used to calculate pharmacokinetics parameters, such as, AUC_{12h} (area under the plasma drug concentration–time curve between 0 and 12 h), extrapolated AUC_{inf} (AUC from 0 to infinity), and $t_{1/2}$ (drug half-life). For bioequivalence analysis, two-way analysis of variance (ANOVA) was performed using the K-BE Test 2002 program at a significant level of 0.05 [20].

3. Results and discussion

3.1. Optimization of MS detection and chromatographic conditions

Mass spectrometric parameters were optimized to achieve the maximum abundance of the product and fragmented ions of the compounds analyzed. The main instrument parameters

Table 1
LC–MS/MS instrument parameters

Parameters	Value
Source temperature (°C)	100
Desolvation temperature (°C)	350
Desolvation gas flow (l/h)	650
Cone gas flow (l/h)	100
Collision cell gas pressure (mbar)	3.46×10^{-3}
Capillary voltage (kV)	3.5
Extractor (V)	3
RF lens (V)	0
Low mass 1 resolution	14
High mass 1 resolution	14
Ion energy 1 (V)	1
Low mass 2 resolution	14
High mass 2 resolution	14
Ion energy 2 (V)	1
Entrance (V)	2
Exit (V)	2
Multiplier	650
Dwell time (s)	0.5

Parameters	Analyte	IS
Molecule ion (*m/z*)	254.00	226.00
Product ion (*m/z*)	152.09	152.09
Cone energy (V)	20	20
Collision energy (V)	15	20

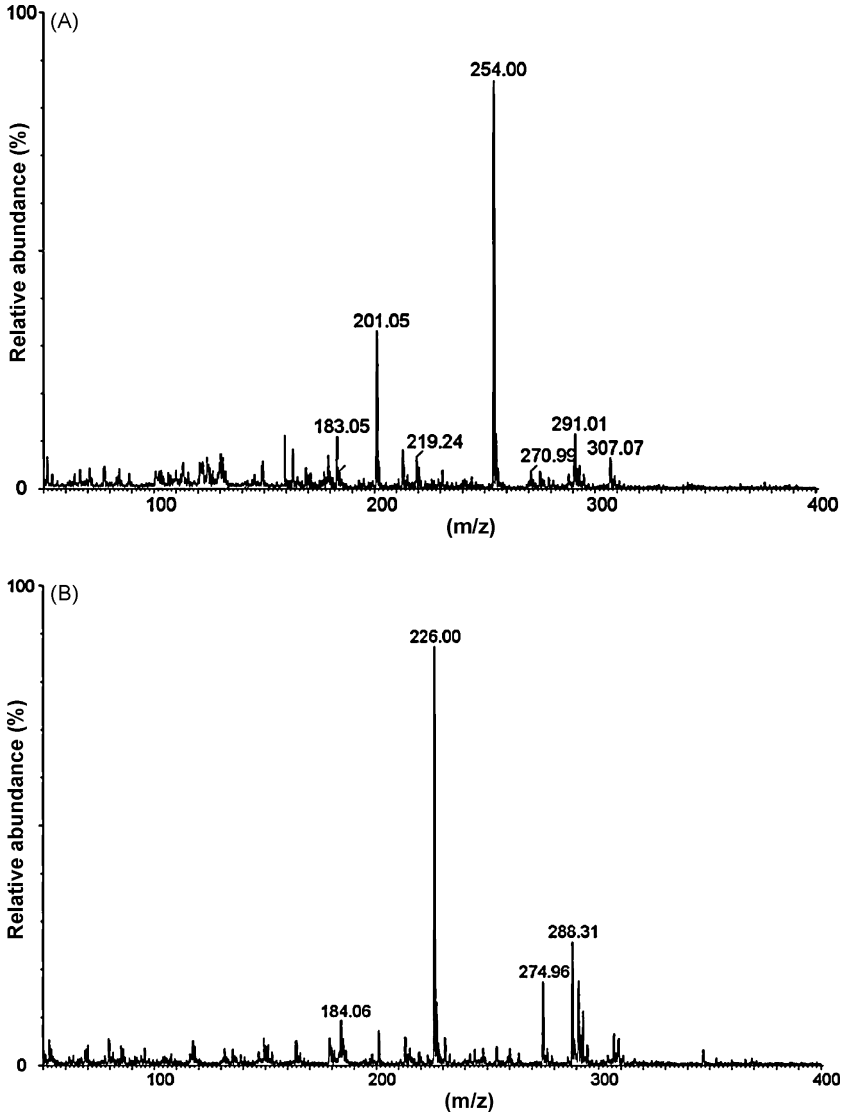

Fig. 2. Full-scan mass spectra of: (A) penciclovir and (B) IS.

of the mass spectrometer are summarized in Table 1. Full scan mass spectra and product ion scan spectra of penciclovir and IS were obtained by direct infusion into the mass spectrometer of 0.1 μg/ml diluted in the mobile phase at a flow rate of 0.2 ml/min. Penciclovir and IS mass spectra exhibit protonated species $[M+H]^+$ at *m/z* 254.00 and *m/z* 226.00, respectively, which were chosen as precursor ions. These compounds fragmented to produce intense product ion signals at *m/z* 152.09 and *m/z* 152.09, respectively. Ionization and fragmentation were found to be highly efficient, and as a result, a substantial detection response was obtained at the lower limit of quantitation (0.05 μg/ml). Full scan and product ion mass spectra of penciclovir and of IS are displayed in Figs. 2 and 3, respectively.

3.2. Sample preparation

To decrease LLOQ and the analysis time, liquid–liquid extraction was performed using five different extraction solvents (hexane, dichloromethane, methyl-*t*-butyl ether, butyl acetate, and ethyl acetate). However, no solvent allowed good recovery of analytes, and thus, deproteination with 100% methanol was used as a fast and straightforward method of sample preparation. Under these conditions, the responses of penciclovir and IS were consistent, precise, and reproducible.

3.3. Validation and matrix effect

Fig. 4 shows the typical chromatograms of a blank plasma sample, a blank plasma sample spiked only with penciclovir, a blank plasma sample spiked only with IS, and a plasma sample from a healthy volunteer 1 h after the oral administration of 500 mg famciclovir (measured penciclovir concentration in plasma: 2.4 μg/ml). Using the described chromatographic conditions, the total analysis time per run was 2 min. Penciclovir and IS were eluted at 0.98 and 0.96 min, respectively. No interferences from constituents of drug-free human plasma were observed, and the selectivity of the method in the presence

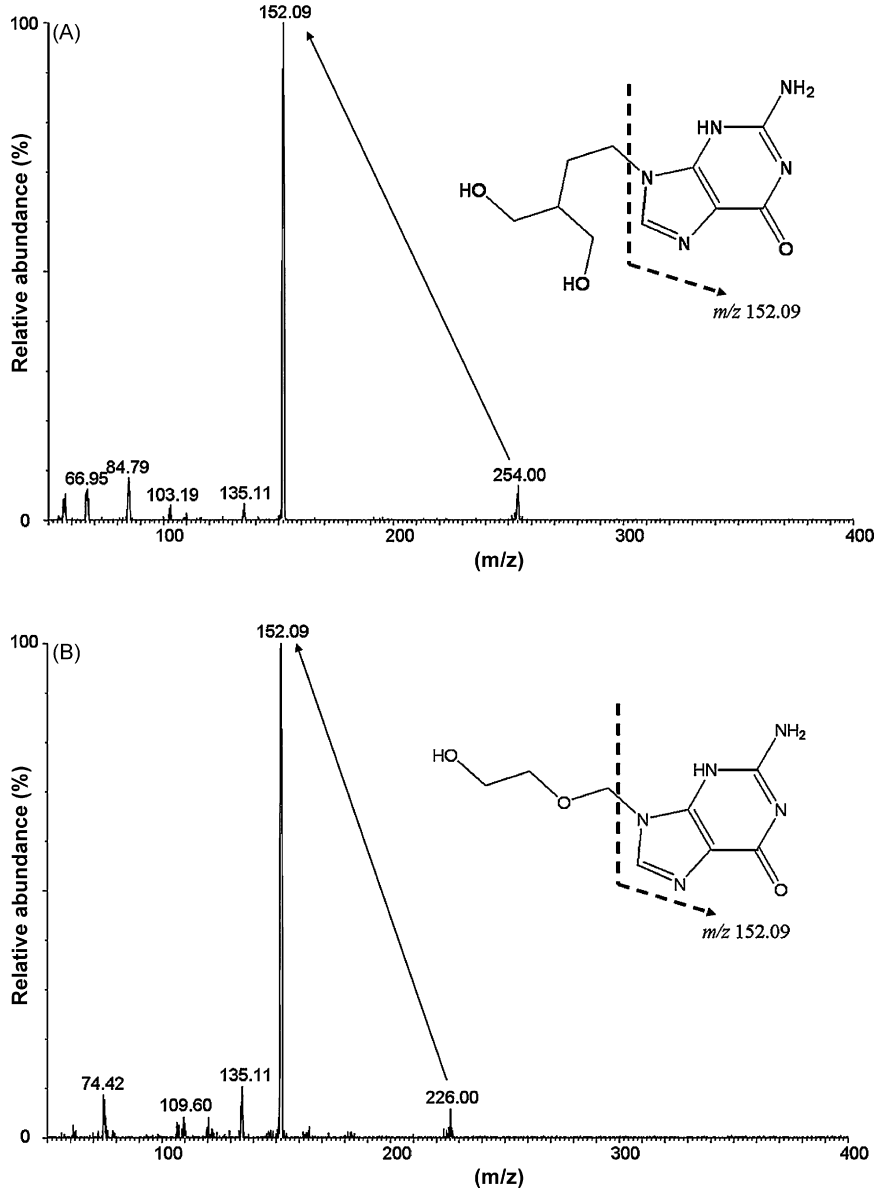

Fig. 3. Product ion spectra of $[M+H]^+$ ions of: (A) penciclovir and (B) IS.

of endogenous plasma compounds was found to be fully satisfactory.

Assay linearity was evaluated using penciclovir calibration curves from 0.05 to 10 μg/ml. A good linear relationship between peak area ratios and concentrations was established: for penciclovir, $y = 0.000224(\pm 0.000002)x + 0.003085$ (± 0.000191); $r^2 = 0.9999(\pm 0.00004)$, where y is the peak area ratio (penciclovir/IS) and x is the concentration (μg/ml) of penciclovir.

The intra-day mean accuracy of the developed method for penciclovir ranged from 2.0 to 8.4% (defined as RE), whereas the intra-day precision ranged from 7.8 to 2.3% (defined as R.S.D.). The inter-day accuracy ranged from 1.9 to 9.1%, and the inter-day precision ranged from 3.7 to 7.5% (Table 2). Intra-day and inter-day accuracy and precision for both analytes were less than 10. All results were within the ranges of precision (%) and accuracy (%) specified by the KFDA guidelines [19].

Table 2
Reproducibility and accuracy for penciclovir in human plasma

Nominal concentration (μg/ml)	Mean found concentration (μg/ml)	Precision, R.S.D. (%)	Mean relative error[a], RE (%)
Intra-day			
0.05	51.1	7.8	2.2
0.5	490.1	3.6	2.0
10	9157.2	2.3	8.4
Inter-day			
0.05	45.4	7.5	9.1
0.5	509.7	4.3	1.9
10	9717.4	3.7	2.8

[a] Mean relative error = |mean measured concentration − added concentration| × 100/nominal concentration.

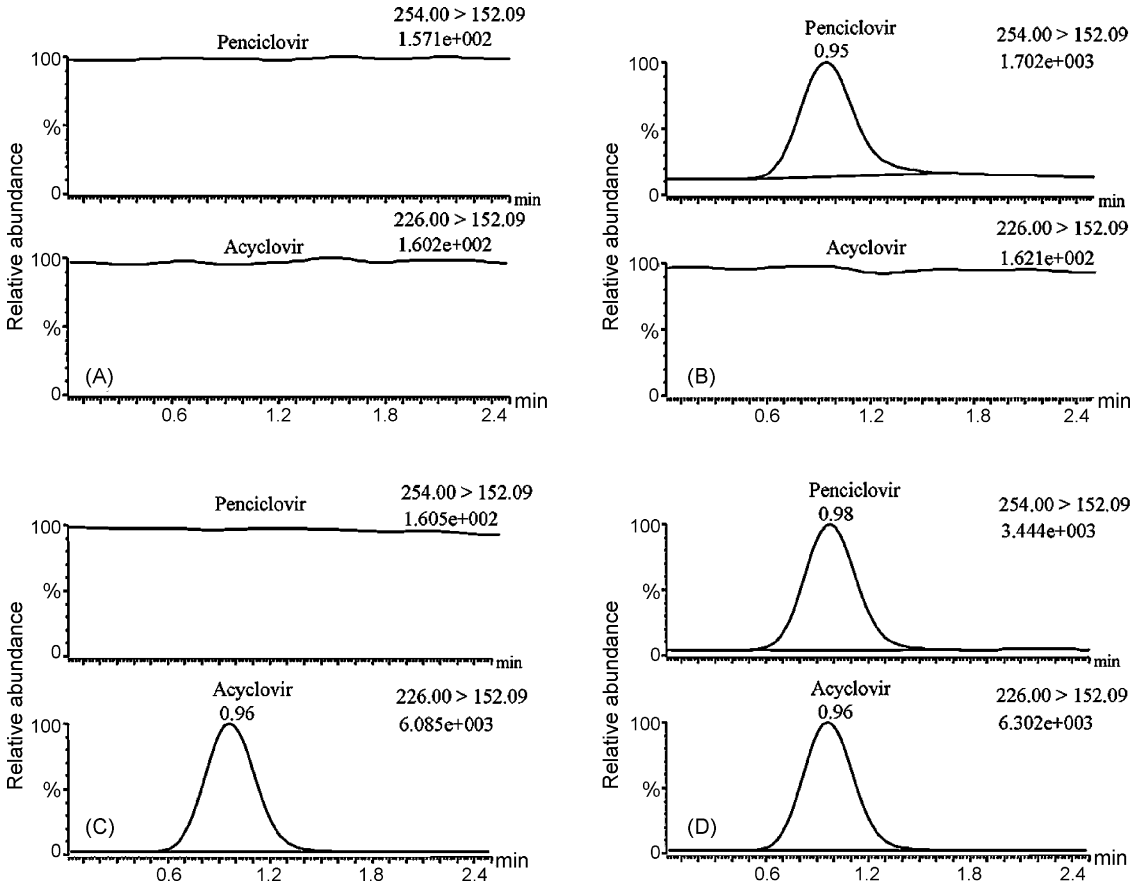

Fig. 4. (A) Blank plasma sample, (B) plasma spiked with 1 μg/ml of penciclovir, (C) plasma sample spiked with IS (0.02 ml, 200 μg/ml) and (D) a volunteer plasma sample 1 h after an oral dose of 500 mg penciclovir (measured penciclovir concentration: 2.4 μg/ml).

The mean matrix effects at three concentrations (0.05, 0.5 and 10 μg/ml) were 112.9, 106.6 and 108.4%, respectively. These results suggest the presence of a minimal matrix effect in terms of the ionization of penciclovir and IS. The R.S.D.s of lot-to-lot matrix variations at three concentrations were 3.6, 6.5 and 2.2%, respectively, suggesting an absence of significant inter-source variability in the matrix effect. Thus, we conclude that the present analytical method is sufficiently reliable and is subject to minimal matrix effect.

3.4. Stability

Studies performed to determine drug stability in plasma and in the mobile phase used for preparing standard solutions showed no degradation. Table 3 lists data for bench (room temperature), auto sampler (4 °C) and freeze–thaw stability of penciclovir. These results indicate that penciclovir was stable under all of the conditions described above, and importantly no stability-related problems are to be encountered during routine sample analysis.

3.5. Application to the clinical test

The proposed method was applied to the determination of penciclovir in plasma samples generated during a bioequivalence study in 24 healthy Korean male volunteers who were orally administered 500 mg of famciclovir in tablet form. High-throughput sample analyses are paramount importance in studies that require a large numbers of samples to be analyzed; as is normally required by pharmacokinetic investigations. The sample preparation described using methanol could resolve this problem. In the present study, 624 clinical samples were divided into two equal batches and each batch consisted of a calibration curve, 312 samples from 12 subjects and QC samples. It took about 0.5 h to prepare 312 samples and 7 h to conduct the analysis. Fig. 5 shows the mean plasma penciclovir concentration–time curves for the two famciclovir tablet

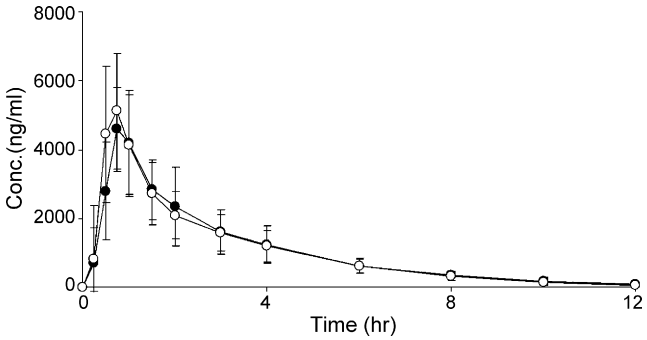

Fig. 5. Mean (±S.D.) concentration–time pharmacokinetic profiles of Famvir tablet (●) and Kukje famciclovir tablet (○) in the plasma samples of 24 healthy male volunteers after a single oral administration of 500 mg of famciclovir.

Table 3
Stability of penciclovir in human plasma and in standard solution ($n = 5$)

Condition tested	QCL (0.05 μg/ml)		QCM (0.5 μg/ml)		QCH (10 μg/ml)	
	R.S.D. (%)	RE (%)	R.S.D. (%)	RE (%)	R.S.D. (%)	RE (%)
Short-term stability						
Control samples (freshly prepared)	7.3	–	5.1	–		–
Freeze–thaw (−80 °C, 3 cycle)	9.7	2.0	2.8	8.3	5.0	0.8
Bench (room temperature, 6 h)	1.0	7.7	3.8	5.9	2.8	4.2
Bench (room temperature, 1 day)	1.0	5.7	4.3	10.4	3.9	7.3
Bench (room temperature, 2 day)	1.2	4.1	4.6	3.5	3.4	5.6
Autosampler (4 °C, 1 day)	1.4	3.1	3.9	2.5	6.2	3.6
Freezer (−80 °C, 1 day)	8.2	7.7	7.4	9.2	6.5	3.2
Sample dilution (1/2)	4.1	10.4	9.7	11.8	5.7	8.9
Sample dilution (1/4)	3.2	9.8	1.8	10.4	2.4	10.2
Mobile phase (room temperature, 12 h)	2.0	9.7	5.3	5.1	6.1	3.3
Long-term stability						
Freezer (−80 °C, 30 day)	4.9	1.0	8.6	7.2	4.8	6.1
Mobile phase (4 °C, 20 day)	5.8	8.0	6.3	0.6	0.5	0.7

Table 4
Bioequivalence parameters obtained after oral administration of Famvir tablet (reference drug) and Kukje famciclovir tablet (test drug) at the famciclovir dose of 500 mg

	Reference drug (mean ± S.D.)	Test drug (mean ± S.D.)	90% confidence interval
C_{max} (μg/ml)	5.04 ± 1.45	5.50 ± 1.78	0.9546–1.0395
AUC_{12h} (μg h/ml)	12.42 ± 3.96	12.34 ± 3.84	1.0119–1.1447
AUC_{inf} (μg h/ml)	12.78 ± 4.04	12.60 ± 3.80	0.9470–1.0364
T_{max} (h)	0.9 ± 0.4	0.7 ± 0.2	–
$t_{1/2}$ (h)	2.27	2.45	–

formulations, and the pharmacokinetic parameters derived from these curves are presented in Table 4. No significant differences were observed between the two formulations in relation to C_{max}, AUC_{12h} and AUC_{inf}.

4. Conclusions

A simple, rapid and sensitive LC–MS/MS method for the determination of penciclovir in human plasma was developed and validated according to KFDA guidelines. The devised LC–MS/MS method has high sensitivity and specificity, and is capable of achieving high sample throughputs, which allows its straightforward use for pharmacokinetic assays, such as bioequivalence study.

Acknowledgements

This work was supported by the Korean Science & Engineering Foundation (grant no. R13-2002-020-01002-0) and by the Seoul Research and Business Development Program (10524).

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