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Determination of penciclovir in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry: application to a clinical pharmacokinetic study

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ABSTRACT: A rapid, specific and sensitive liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed for the determination of penciclovir in human plasma. The method involved simple, one-step SPE procedure coupled with a C_{18}, 75 × 4.mm, 3μm column with a flow-rate of 0.5 mL/min, and acyclovir was used as the internal standard. The Quattro Micro mass spectrometry was operated under the multiple reaction-monitoring mode using the electrospray ionization technique. Using 250 μL plasma, the methods were validated over the concentration range 52.555–6626.181 ng/mL, with a lower limit of quantification of 52.55 ng/mL. The intra- and inter-day precision and accuracy values were found to be within the assay variability limits as per the FDA guidelines. The developed assay method was applied to a clinical pharmacokinetic study in human volunteers. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: penciclovir; pharmacokinetic studies; LC-MS/MS; bioanalysis; human plasma

Introduction

Herpes virus 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* (Boyd *et al*., 1987, 1988a, b). Moreover, penciclovir is a potent and highly selective inhibitor of herpes viruses, such as herpes simplex virus types 1 and 2, varicella-zoster virus, Epstein virus and hepatitis B virus (Harnden *et al*., 1987; Sutton and Boyd 1993; Korba and Boyd 1996). It has been demonstrated that, in ocular herpes zoster, penciclovir reduced the incidence of ocular complications (Tyring *et al*., 2001), but ocular penetration and pharmacokinetics of penciclovir in the human eye are still unknown. However, penciclovir has poor bioavailability when administered orally to human (Boyd *et al*., 1988a, b). 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 (Harnden *et al*., 1989; Vere Hodge *et al*., 1989; Pue and Benet, 1993).

After oral administration, famciclovir is converted to penciclovir by the removal of two ester groups and the 6-oxidation of the deoxyguanine ring. Four intermediate metabolites, depending on the rate of de-esterefication and oxidation, are obtained (Vere Hodge *et al*., 1989; McMeekin *et al*., 1992), but pharmacokinetic studies have shown that penciclovir, and to a minor extent BRL42355, the desacetyl non-oxidized metabolite, are predominant in plasma and urine (Filer *et al*., 1994, 1995).

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 analogs in biological fluids (Loregian *et al*., 2001). In previous pharmacokinetic studies (Pue *et al*., 1994; Boike *et al*., 1994), penciclovir was analyzed by high-performance liquid chromatography (HPLC) with UV (Kim *et al*., 1999; Fowles and Pierce 1989; Pue *et al*., 1994) or fluorescence detection (Schenkel *et al*., 2005; Zhou *et al*., 2006). 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 sample preparation and large biological volumes (i.e. up to 0.5 mL of plasma). Nowadays, liquid

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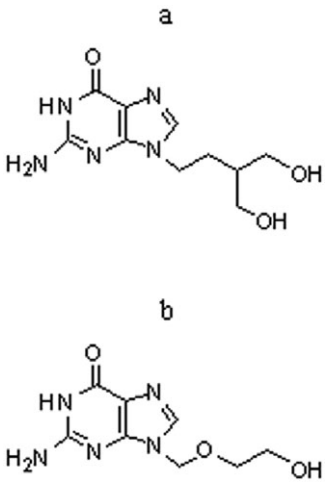

Figure 1. Structure of Penciclovir and Acyclovir.

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 date only one LC–MS/MS procedure has been reported for the quantitation of penciclovir (Lee *et al*., 2007), which employed protein precipitation to extract penciclovir from plasma. The method is sensitive but the sample preparation method is not preferred for mass-spetrometry due to the matrix effect; this may be a limiting factor when it is applied to large number of subject samples. This paper describes a novel and selective approach, which enables the determination of penciclovir with good accuracy at low drug concentrations in plasma using liquid chromatography coupled to tandem mass spectrometry (MS/MS). This method was fully validated and applied to a bioequivalence study in healthy volunteers after oral administration of 500 mg tablets of famciclovir.

Experimental

Chemicals and Reagents

The working standards of Penciclovir and Acyclovir (Fig. 1) were obtained from Synchron Research Services Pvt. Ltd (Ahmedabad, India). High-purity water was prepared in-house using a Milli-Q water purification system obtained from Millipore (Bangalore, India). HPLC methanol, acetonitrile and hydrochloric acid were purchased from J. T. Baker (USA) and formic acid was purchased from Merck (Germany). Drug-free (blank) human plasma was obtained from Prathma Laboratory (Ahmedabad, India) and was stored at −20°C prior to use.

Calibration Curves

The stock solution of Penciclovir was prepared in water at a concentration of 1000 μg/mL with the aid of concentrated HCl and internal standard (acyclovir) was prepared in water at a concentration of 250 μg/mL with the aid of concentrated HCl. These diluted working standard solutions were used to prepare the calibration curve and quality control samples. A nine-point standard calibration curve for penciclovir was prepared by spiking 250 μL blank plasma with 5 μL of an appropriate working standard solution. The calibration curve range was 52.555–6626.181 ng/mL. Quality control samples were prepared at three concentration levels of 154.014 ng/mL (low), 2916.925 ng/mL (medium) and 5303.501 ng/mL (high).

Sample Preparation

A 250 μL aliquot of human plasma sample was mixed with 25 μL of internal standard working solution (250 μg/mL of acyclovir) and vortexed for 30 s. The samples were transferred to a 1 cm^3/30 mg Oasis HLB SPE column, which had been conditioned with 1.0 mL methanol, followed by 1.0 mL water. After application of the samples, the SPE column was washed with 1.0 mL of MilliQ water and dried for 1.0 min by applying positive pressure at maximum flow rate. The column was eluted with 1.0 mL of the reconstituted solution (acetonitrile–MilliQ water, 4:1, v/v buffered with 0.2% formic acid) and vortexed for about 10 s. The SPE elutes were transferred into 1 mL LC vials for injection of 5 μL into the LC system

Instrumentation

The chromatography was performed on a Quattro Micro system (Waters corp., Milford, MA, USA) with cooling auto-sampler and column oven enabling temperature control of the analytical column. The Purospher star C_{18} column (75 × 4.mm, 3 μm; Merk, Germany) was employed. The column temperature was maintained at 45°C and chromatographic separation was achieved using mobile phase consisting of acetonitrile–water–formic acid (80:20:0.2, v/v/v) and delivered with a flow rate of 0.5 mL/min. The auto-sampler was maintained at 10°C and the injection volume was 5 μL. Total run time for each sample analysis was 2.5 min.

Validation

The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation (FDA 2001). The method was validated for selectivity, linearity, precision, accuracy, recovery and stability. Selectivity was assessed, by comparing the chromatograms of six different batches of blank plasma obtained from six different sources (or donors) with those of corresponding standard plasma samples spiked with penciclovir and IS. Sensitivity was determined by analyzing six replicates of blank human plasma and plasma spiked with lowest level of the calibration curve. All validation experiments were carried out at three QC levels. For the determination of intraday accuracy and precision, a replicate ($n = 6$) analysis of plasma samples was performed on the same day. The inter-day accuracy and precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (RSD%) and the accuracy as the relative error (RE%). Recovery of penciclovir and IS was determined by comparing the peak area of extracted analyte standard with the peak area of non-extracted standard. The matrix of plasma constituents over the ionization of analyte and IS was determined by comparing the responses of the post-extracted plasma standard QC samples ($n = 6$) with the response of analyte from neat samples at equivalent concentrations (Matuszewski *et al*., 2003; Dams *et al*., 2003; Viswanathan *et al*. 2007). The matrix effect was determined at two levels (LQC and HQC) for penciclovir and for IS at 6.25 μg/mL. Dilution integrity was performed to extend the upper concentration limit with acceptable precision and accuracy. Process sample stability was evaluated by re-injecting the same sample with freshly spiked calibration curve and quality control samples, which (stability samples) were stored at 10°C for 46 h. Bench-top stability was evaluated for 6 h and compared with freshly spiked plasma samples. The freeze–thaw stability was determined by comparing the stability samples that had been frozen and thawed three times with freshly spiked quality control samples. Long-term stability was evaluated by analyzing LQC and HQC samples stored at −20°C for 60 days together with freshly spiked calibration and quality control standards. All stability evaluations were based on back calculated concentrations. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. 15% RSD).

Pharmacokinetic Study

The method was applied to determine the plasma concentrations of penciclovir from a bioequivalence study in which 12 healthy male volunteers

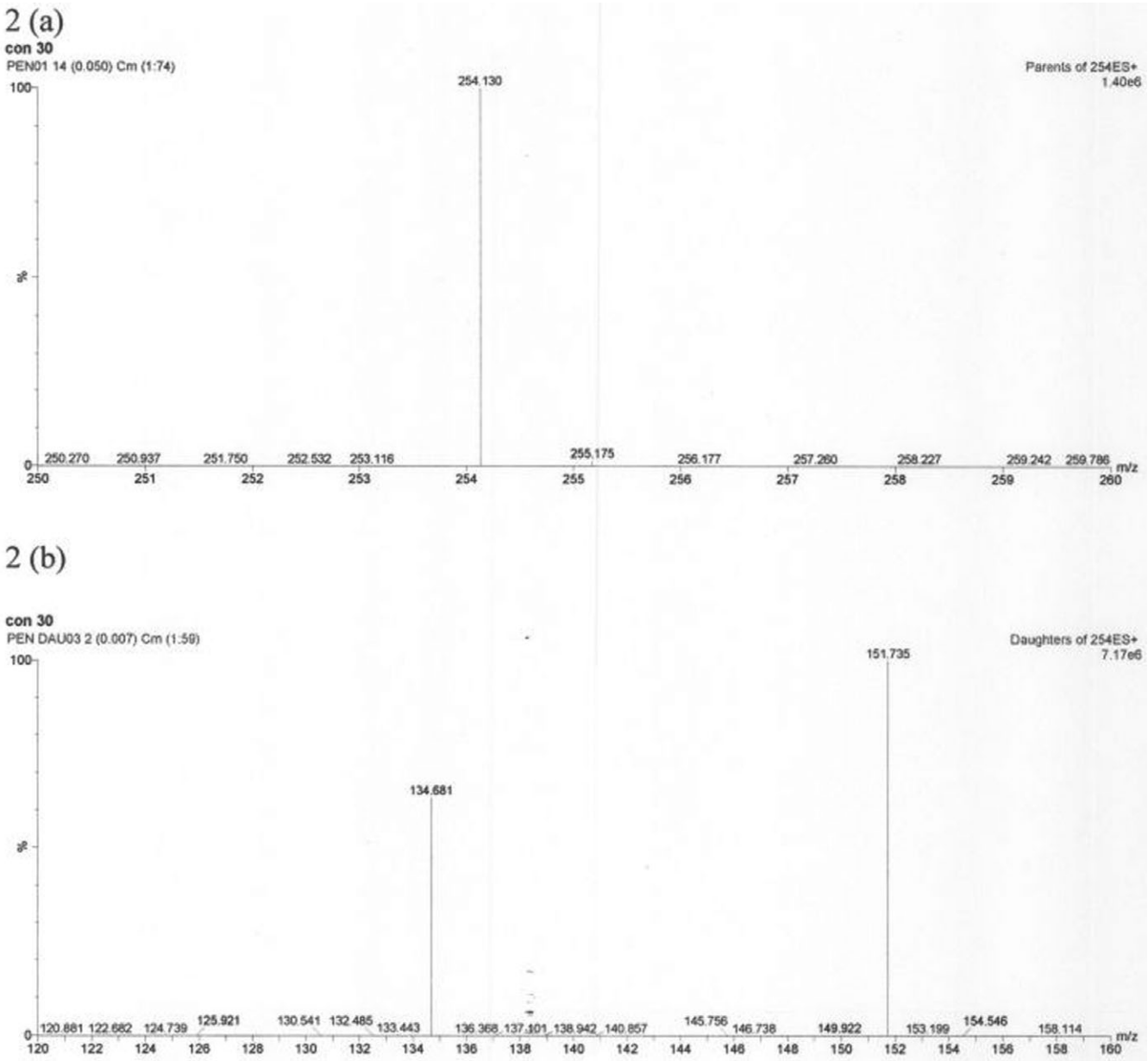

Figure 2. (a) Q1 Scan of Penciclovir and (b) MS/MS Scan of Penciclovir; (c) Q1 Scan of IS and 2(d) MS/MS Scan of IS.

received two tablets (containing 500 mg famciclovir). The ethics committee approved the protocol and the volunteers provided with informed written consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were obtained following oral administration of 500 mg famciclovir tablet into K_3EDTA vacutainer solution as an anticoagulant at pre-dose, 0.17, 0.33, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, 2.50, 3.00, 3.5, 4.00, 5.00, 6.00, 8.00, 10.00, 12.00 and 14.00 h. Plasma was harvested by centrifuging the blood using an Eppendorf centrifuge 5810R (Eppendorf, Germany) at 3000 rpm for 5 min and stored frozen at −20 ± 10°C until analysis. An aliquot of 250 µL of thawed plasma samples were spiked with IS and processed. Plasma concentration–time data of penciclovir was analyzed by non-compartmental method using Kinetica Version 4.4 (Thermo USA).

Results and Discussion

Chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and increase the signal of analytes, as well as short run time. The presence of a small amount of penciclovir in the mobile phase improved the detection of the analytes. It was found that a mixture of acetonitrile–formic acid (0.2%; 80:20 v/v) could achieve this purpose and this was finally adopted as the mobile phase. Two channels were used for recording, channel 1 for penciclovir with a retention time about of 1.00 min and channel 2 for acyclovir with retention about time of 1.03min. Penciclovir and IS were rapidly eluted with retention time less than 1.5 min, and total run time was just 2.5 min per sample; this met the requirement for a high sample throughput.

Mass Spectrometry

In order to optimize ESI conditions for penciclovir and IS, quadrupole full scans were carried out in positive ion detection mode.

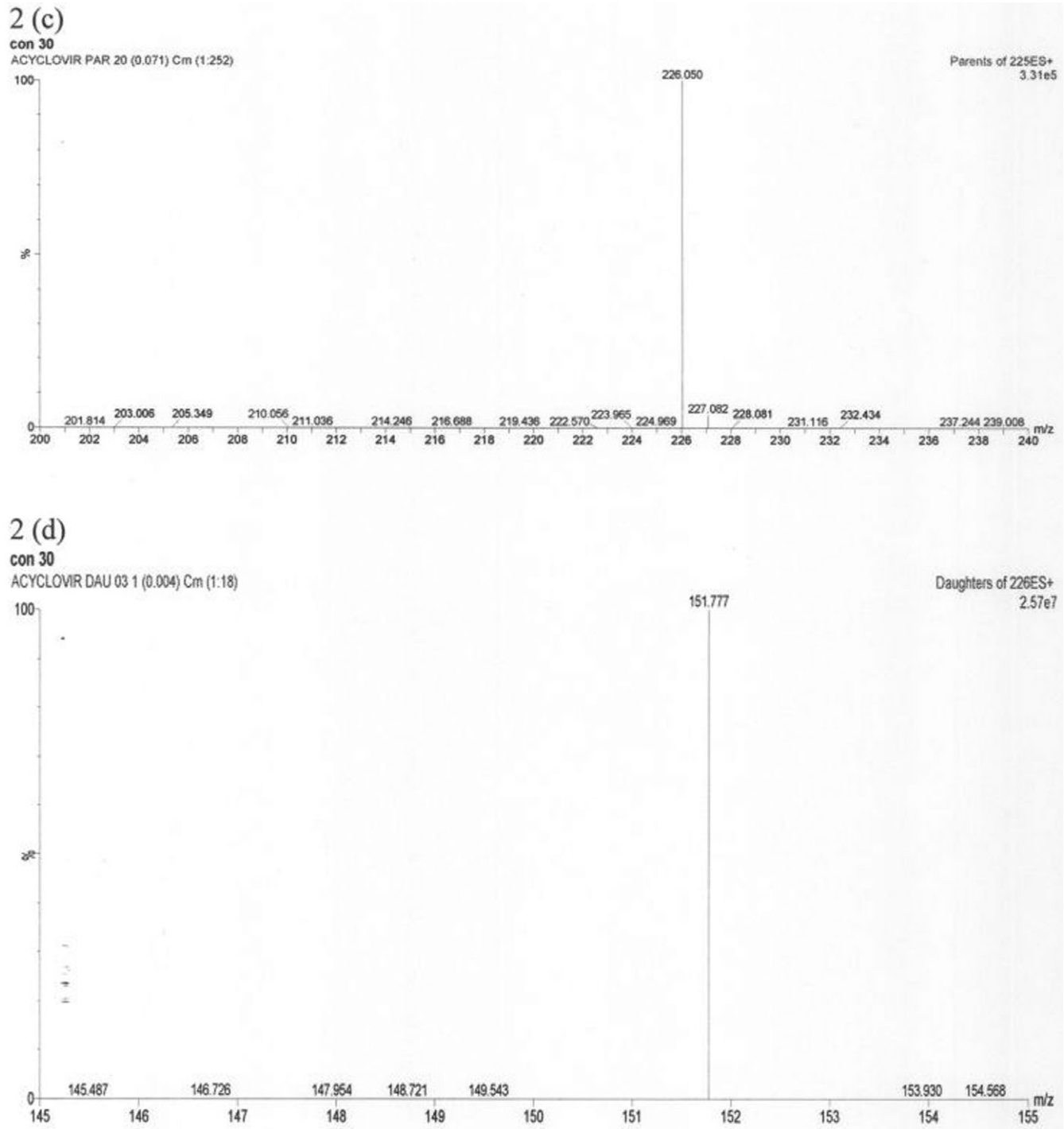

Figure 2. *Continued.*

During a direct infusion experiment, the mass spectra for penciclovir and IS revealed peaks at *m/z* 254 and 226, respectively; parent ions and the daughter ions at 152 and 152 are depicted in Fig. 2. The analysis temperature, nebulizer gas and ESI temperature were investigated to optimize the specificity and sensitivity of ions detection. Ions were detected in multiple reaction monitoring mode: the transition parts of penciclovir at the *m/z* 254 *amu*, precursor ions to the *m/z* 152 *amu* product ion *m/z* 226 *amu* precursor ion to the *m/z* 152 *amu* product ion for the IS. Q1 and Q3 were referenced at unit resolution. The analytical data were processed by Maslynx 4.0.

Selectivity

The selectivity of the method was examined by analyzing ($n = 6$) blank human plasma extract against plasma spiked with the lowest standard. As shown in Fig. 3, no significant direct interference in the blank plasma traces was observed from endogenous

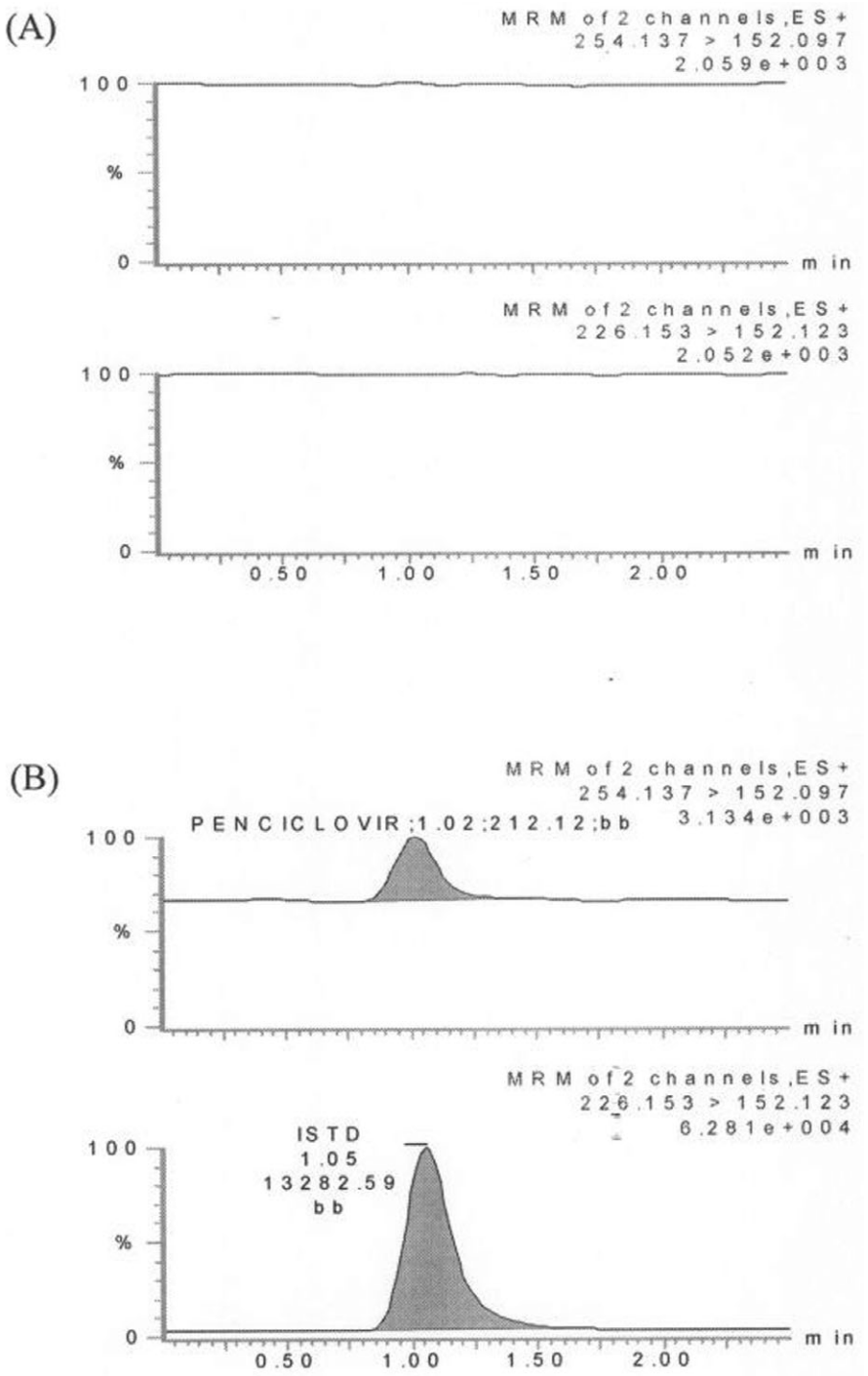

Figure 3. Representative chromatograms of (A) Extracted blank plasma sample; (B) Extracted lower limit of quantification plasma sample.

substances in drug-free human plasma at the retention time of the analyte.

Linearity

A nine-point calibration curve was found linear over the concentration range 52.555–6626.181 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.99; Table 1 summarizes the calibration curve results.

Sensitivity

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and

Table 1. Precision and accuracy data of back-calculated concentration of calibration samples for Penciclovir in human plasma

Concentration added (ng/mL)	Concentration found (mean:ng/mL)	Precision (%)	RE(%)
52.555	54.068	2.8	2.9
105.110	99.453	5.4	−5.4
2:0.221	197.648	5.7	−6
323.417	349.401	6.3	8
980.052	992.198	11.7	1.2
3266.840	3012.079	3.6	−7.8
4949.75?	5029.638	7.1	1.6
5963.563	6088.451	3.3	2.1
6626.181	6844.982	7.5	3.3

Table 2. Precision and accuracy of the method for determining Penciclovir concentration in plasma samples

Concentration added (ng/mL)	Intra-day precision (n = 6)			Inter-day precision (n = 18)		
	Concentration found (mean; ng/mL)	Precision (%)	RE(%)	Concentration found (mean; ng/mL)	Precision (%)	RE(%)
154.014	142.877	4.9	−7.2	150.362	8.8	−2.4
2916.925	2609.272	4.5	−10.5	2733.079	10.2	−6.3
5303.501	5034.842	0.7	5.1	5078.146	11.7	−4.2

precision, and it was found to be 52.555 ng/mL. The intra-day precision at the LLOQ was 2.8 % and accuracy was 2.9%.

Precision and Accuracy

The results for intra-day and inter-day precision and accuracy in plasma quality control samples are summarized in Table 2.The intra-day precision ranged from 0.7 to 4.9% and accuracy ranged from −10.5 to 5.1%. The inter-day precision ranged from 8.8 to 11.7% and accuracy ranged from −6.3 to −2.4%.

Recovery

Five replicates at low, medium and high quality control concentrations were prepared for recovery determination. The mean recovery was 54.0 and 47.8% for penciclovir and acyclovir, respectively.

Matrix Effect

The matrix effect was evaluated by analyzing QC samples. Average matrix factor values (matrix factor = response of post-spiked concentrations/response of neat concentrations) obtained for penciclovir were +0.98 (CV 4.8%, $n = 6$) at LQC and +0.96 (CV 5.9%, $n = 6$) at HQC level, whereas on IS it was found to be +1.00 (CV 7.5%, $n = 6$) at the tested concentration of 6.25 µg/mL. The matrix effect was not observed at analyte and IS retention times.

Dilution Integrity

The upper concentration limits can be extended to 10772.736 ng/mL by a 2-fold or 5-fold dilution with human plasma with a precision of 0.5% and an accuracy of 9.6%.

Stability

The stability of the analyte in human plasma under different temperature and timing conditions was evaluated as follows and the results of the stability studies are enumerated in Table 3. QC samples were subjected to long-term storage conditions (−20°C), and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels (154.014 and 5303.501 ng/mL as LQC and HQC values) with four determinations for each. For process stability, the results indicated that the differences in the back-calculated concentrations from time 0 to 46 h was −6.90 and 0.81%, which allowed us to conclude that processed samples are stable at least for 46 h at 10°C in the autosampler. For bench-top stability, the results allowed us to conclude that both analytes are stable for at least 6 h at room temperature in plasma samples. Freeze and thaw stability results indicated that the repeated freeze and thawing (three cycles) did not affect the stability. Long-term stability of the analytes in plasma at −20°C was found to be good for at least 60 days at −20°C.

Pharmacokinetic Study

The proposed method was applied to determine penciclovir levels in plasma for a bioequivalence study in 12 healthy Indian male volunteers who were orally administered 500 mg of famciclovir in tablet form. High-throughput sample analysis is of particular importance for studies that require the analysis of large numbers of samples, and the described SPE method for sample preparation is suitable for this purpose. Figure 4 shows the mean plasma concentration–time curves for the two formulations. The mean maximum plasma concentration (C_{max}; 4.876 and 3.780 µg/mL for test and reference formulations, respectively) and the T_{max} were found to be ~0.71 h for test and ~1.38 h for reference. The half-life ($t_{1/2}$) was found to be ~2.307 and ~2.436 for test and reference. The AUC_{0-t}, AUC_{0-inf} for test and reference formulations were found to be 13.014 and 13.276 µg h/mL for test and 11.82 and 12.08 µg h/mL for reference. No significant differences were observed between the two formulations in terms of; C_{max}, AUC_{0-t}, AUC_{0-nf}, T_{max} or $t_{1/2}$ (Table 4).

Table 3. Stability samples results for Penciclovir

Stability	n	Spiked Concentration (ng/mL)	Mean calculated comparision sample concentration (ng/mL)	Mean calculated stability sample concentration (ng/mL)	Mean percentage change
Process[a]	4	154.014	167.705	166.344	0.81
	4	5303.501	5165.130	5548.362	–6.90
Bench top[b]	4	154.014	167.705	167.415	0.17
	4	5303.501	5165.130	4756.449	8.59
Freeze and thaw[c]	4	154.014	171.113	159.488	7.2
	4	5303.501	5317.914	5250.617	1.01
Long-term[d]	4	154.014	169.006	153.371	–7.48
	4	5303.501	5263.766	4788.219	–9.03

[a] After 46 h in autosampler at 10°C; [b] after 6 h at room temperature; [c] after three freeze and thaw cycles at –20°C; [d] at –20°C for 60 days.

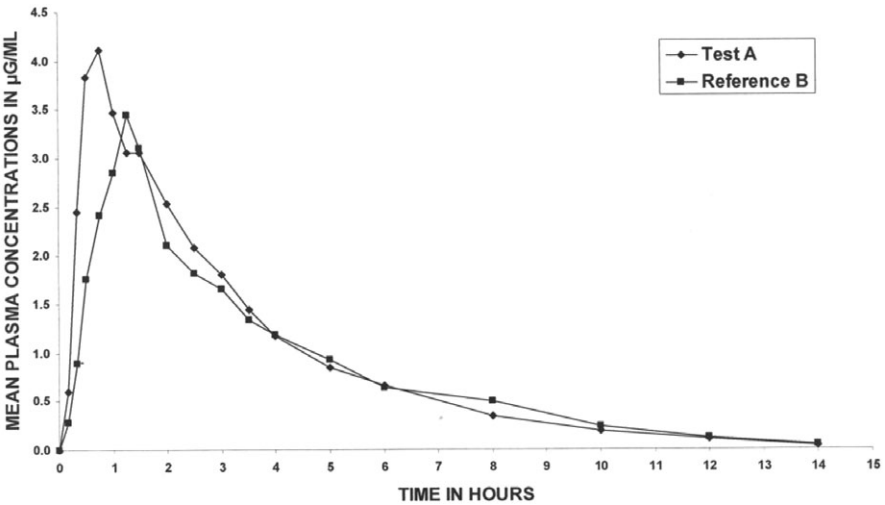

Figure 4. Mean plasma concentration-time profile of Penciclovir in human plasma following oral dosing of 500 mg Famciclovir tablet to 12 subjects.

Table 4. Pharma cokinetic data of PCV in human plasma

Parameter	Test (mean ± SD)	Reference (mean ± SD)
AUC 0-t (µg h ml^{-1})	13.014 ± 2.22	11.819 ± 2.04
AUC 0-inf (µg h ml^{-1})	13.276 ± 2.29	12.081 ± 2.02
Cmax (µg ml^{-1})	4.876 ± 1.33	3.780 ± 1.10
Tmax (µg ml^{-1})	0.71 ± 29	1.38 ± 0.52
Kel (1/h)	0.304 ± 0.036	0.316 ± 0.14
*t*1/2(h)	2.307 ± 0.29	2.436 ± 0.69

Conclusion

A simple, specific, rapid and sensitive LC-MS/MS method has been developed for the determination of penciclovir in human plasma and fully validated according to FDA Guidelines' accepted criteria. Compared with the published methods, the smaller injection volume is of particular advantage when coupled with electrospray mass spectrometry, reducing ion suppression and offering superior sensitivity. Lower limits of detection, higher sensitivity, satisfactory selectivity and a short run time of 2.5 min were observed and its utility was demonstrated successfully in a clinical pharmacokinetic study. From the results of all the validation parameters, we can conclude that the present method can be useful for clinical pharmacokinetic studies.

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