HPLC Method for the Determination of Penciclovir in Human Plasma and Application to a Bioequivalence Study

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Abstract

A simple, specific, and accurate high-performance liquid chromatographic method, using UV detection for the determination of penciclovir in human plasma, is described. Chromatographic separation is performed on a BDS-C_{18} column using a mixture of phosphate buffer (20mM, pH adjusted to 7.5 with phosphoric acid), methanol, and acetonitrile (94:3:3, v/v/v) as mobile phase. The wavelength of the UV detector is set at 254 nm. The flow-rate is 1.0 mL/min. The assay is linear over the concentration range of 0.1–5.0 µg/mL for penciclovir (r > 0.9996). The limit of quantitation for penciclovir in human plasma is 0.1 µg/mL. The relative standard deviation is less than 7.0% for all the analytes. The method is successfully applied to a randomized crossover bioequivalence study of two different famciclovir capsules in 20 healthy volunteers.

Introduction

Penciclovir, the antiviral agent, inhibits herpes virus infection, both in vitro and in vivo (1,2). Penciclovir (Figure 1A) possess a highly selective inhibitor against various herpes viruses, including herpes simplex virus types 1 and 2, varicella-zoster virus, and Epstein-bar virus (3–6). Because of penciclovir's poor bioavailability, famciclovir, the diacetate ester of the 6-deoxy derivative of penciclovir, was developed as a prodrug of penciclovir (7–10). After orally administering famciclovir to humans, more than half of an oral dose of famciclovir was found to be absorbed and converted rapidly to penciclovir (7). It was also found that famciclovir could not be detected in plasma due to extensive metabolism, and pharmacokinetic studies have shown that penciclovir is predominant in plasma and urine (11). Therefore, famciclovir was quantitated by penciclovir in a bioequivalence study of famciclovir capsules.

Several high-performance liquid chromatography (HPLC) methods for the quantification of penciclovir in biological samples have been reported. These methods have involved HPLC coupled with UV (12–14) or fluorescence detection (15). Capillary electrophoresis and HPLC–tandem mass spectrometry (MS–MS) have also been published for the determination of the drug in biological fluids (16,17). However, most of these methods are relatively tedious and time-consuming because of extensive sample preparation steps, such as solid-phase extraction. Some of the utilized methods are not appropriate for routine use, because the necessary equipment is not common, such as HPLC–MS–MS. The capillary electrophoresis technique suffers from low sensitivity, in spite of its high selectivity, efficiency, and fast separation time.

The purpose of the present study was to develop a method which would allow the determination of penciclovir in human plasma using the relatively simple and inexpensive approach of LC with UV detection. We developed an HPLC method for determining penciclovir in human plasma combining a simple precipitating protein procedure with perchloric acid. Less sensitivity was obtained compared to previously reported HPLC methods with MS detection; however, the resulting limit of quantitation (LOQ) (0.1 µg/mL) was sufficient for human pharmacokinetic studies. Thus, this method is suitable for the routine analysis of large batches of biological samples. Finally, the developed and validated method was successfully applied in a bioequivalence investigation of two different famciclovir capsules in 20 healthy Chinese volunteers.

Experimental

Materials and reagents

A famciclovir test capsule (125 mg per capsule) was provided by DiSha pharmaceutical companies (Weihai, China). A reference capsule (125 mg per capsule) was purchased from DaLian MeiLuo (Dalian, China). Penciclovir (purity 99.7%) and acyclovir [Figure 1B, the internal standard (IS) purity 99.5%] were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

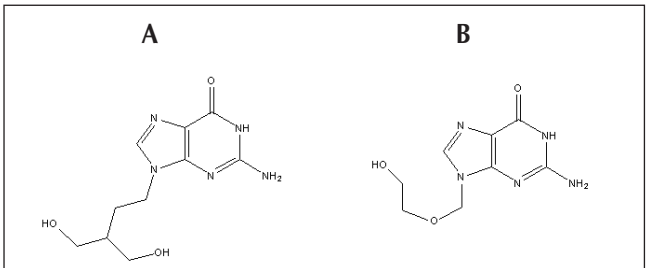

Figure 1. Chemical structures of penciclovir (A) and acyclovir (B).

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Methanol and acetonitrile were of HPLC grade and were obtained from Corncord Tech (Tianjin, China). All other chemicals and reagents were of analytical grade and were purchased from Corncord Tech.

Chromatographic system

The LC system consisted of an LC-10A system (Shimadzu, Kyoto, Japan) equipped with an LC-10AT pump, a fixed injection-loop of 20 µL, a UV detector model SPD-10A UV–vis operated at a wavelength of 254 nm, and a data processor from Anastar (Tianjin, China). The analytical column employed was a Century BDS-C_{18} column (200 × 4.6 mm i.d., 5 µm) with a guard column (10 × 4.6 mm i.d., 5 µm) of the same packing material and purchased from Dalian Zhonghuida Scientific Instrument Co., Ltd. (Dalian, China). The guard column was routinely changed to avoid excessive pressure built-up in the system. The mobile phase was comprised of phosphate buffer (20mM, pH adjusted to 7.5 with phosphoric acid), methanol, and acetonitrile (94:3:3, v/v/v). Flow rate was set at 1 mL/min.

Preparation of standards and quality control samples

Stock standard solutions of penciclovir (100 µg/mL) and acyclovir (500 µg/mL) were prepared with water and stored at 4°C. Penciclovir stock solution was further diluted with water to obtain the working standard solutions ranging from 0.5 to 25.0 µg/mL. Working standard solution of acyclovir was prepared by diluting the stock solution with water to yield a final concentration of 12.2 µg/mL. Plasma calibration samples were prepared using 0.5 mL of drug-free human plasma and 100 µL of different working standard solutions of penciclovir, which finally provided plasma standards with a concentration range from 0.1 to 5.0 µg/mL. Quality control (QC) samples were prepared at low (0.1 µg/mL), medium (1.0 µg/mL), and high (5.0 µg/mL) concentrations in the same way as the plasma samples for calibration.

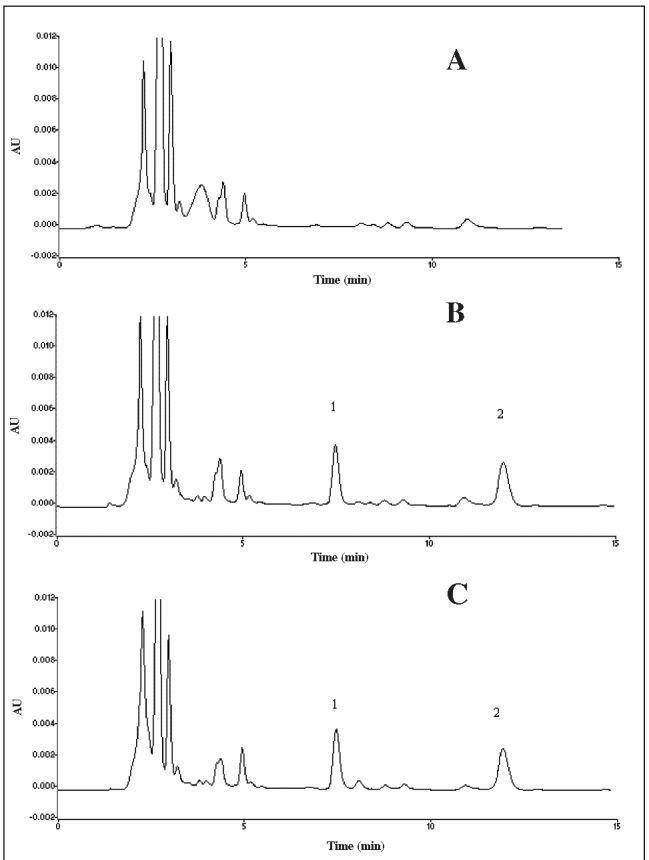

Figure 2. Representative chromatograms of blank plasma (A), plasma spiked with penciclovir (2.5 g/mL) and IS (B), and a plasma sample obtained at 0.75 h after a single oral dose of 250 mg famciclovir (C); IS (peak 1) and penciclovir (peak 2).

Preparation of plasma sample

Blood samples were collected in tubes containing heparin. After centrifugation (10 min at 3000 × *g*), the separated plasma was collected and stored at –20°C. Before analysis, the plasma samples were thawed at 4°C. One hundred microliters of the IS working solution (acyclovir, 12.2 µg/mL) were added to 0.5 mL of plasma sample except for the blank plasma sample. Then, 100 µL of 14% perchloric acid were added to precipitate protein in plasma. After mixing for 1 min on a vortex mixer, and centrifugation (8 min at 8000 × *g*), the supernatant of 20 µL was injected into the HPLC system.

Pharmacokinetic calculations and statistical analysis

The pharmacokinetic parameters, namely the maximum plasma concentration (C_{max}) and time point of maximum plasma concentration (T_{max}) were obtained directly from the measured data; half-life of drug elimination during the terminal phase ($T_{1/2}$), area under the plasma concentration-time curve from 0 to the last measurable concentration (AUC_{0-t}), and area under the plasma concentration-time curve from 0 to infinity ($AUC_{0-\infty}$) were computed using 3P97 computer program (Chinese Society of Mathematical Pharmacology, China). Statistical significance was defined at the level of $P < 0.05$.

Results and Discussion

Selection of IS

IS was applied to get high accuracy when penciclovir was determined in human plasma. Ganciclovir and acyclovir, like penciclovir, are nucleoside analogues that share structural similarity with endogenous compounds. Ganciclovir and acyclovir were investigated to find the suitable one. Ganciclovir was used as the IS in previously reported studies (15). However, it was impossible to separate ganciclovir from the endogenous interference and the retention time was not optimal in the presented method. Based on pilot investigations, acyclovir was chosen as the internal standard because it was completely separated from the endogenous interference and the whole analysis time was short. This suitable internal standard was useful to correct volumetric errors and to improve linearity and selectivity of the detection system.

Selection of plasma sample preparation method

Sample preparation plays an important role in the determination of drugs in biological samples. Various techniques were investigated for the extraction of penciclovir from human plasma. Because penciclovir and acyclovir both possess good solubility in water, conventional liquid–liquid extraction could

not be used for the extraction of samples from plasma. Solid-phase extraction is a well-established sample preparation technique in bioanalytical applications (15). However, the technique is relatively tedious, time-consuming, and expensive. Therefore, the precipitating protein method was evaluated. In order to find the suitable protein precipitation agent, the effects of three kinds of precipitating solvents on recoveries and peak shapes were studied. The solvents methanol and acetonitrile are not suitable for precipitation, because their recoveries are too low and the chromatographic peaks are poor. Perchloric acid as precipitant could make protein fully precipitate using only a small volume, and could make the LOQ of penciclovir attain 0.1 µg/mL. Perchloric acid in different concentrations was added to precipitate protein. The use of a lower amount of perchloric acid resulted in incomplete precipitation; however, high amounts may reduce chromatographic column lifetime. Thus, 14% perchloric acid was selected as the protein precipitation agent.

Selection of mobile phase

The composition of mobile phases was optimized through several trails to achieve good resolution and symmetric peak shapes for penciclovir and IS, and to achieve a short time in the experiment. The use of the buffer in the mobile phase ensured the stable chromatographic retention time of penciclovir and IS. Because of 14% perchloric acid used in sample preparation, phosphate buffer in the mobile phase is necessary for buffering the acidic property of perchloric acid. Changing the proportion of methanol did not separate acyclovir and the endogenous substance. In this experiment, different concentrations of acetonitrile were added to the mobile phase containing 3% methanol. The results indicated that acyclovir, penciclovir, and endogenous materials were separated completely in a mixture of 3% acetonitrile, 3% methanol, and buffer solution. It was also found that the pH of the buffer had significant influence on the chromatographic separation. Therefore, the effect of pH value on separation was investigated for the further optimization of the chromatographic conditions. An increase of pH value from 5.0 to 8.0 caused an increase in the retention times of the two analytes. The resolution of penciclovir and endogenous interferences further increased at pH 7.5. This chromatography condition satisfied the requirement of determination and the retention times of two analytes were optimum. The pH 7.5 was selected as the best resolution condition for the reason that penciclovir and acyclovir were alkaline, and with an increase in pH, the retention times of penciclovir and acyclovir also increased; however, the retention times of endogenous materials almost did not change. Thus, this situation made resolution increase. From these findings, pH 7.5 was selected as optimum pH for the separation. Under the optimum mobile phase conditions, peak shape and resolution of analytes were satisfactory. Finally, a mixture of phosphate buffer (20mM, pH adjusted to 7.5 with phosphoric acid), methanol, and acetonitrile (94:3:3, v/v/v) was selected as the mobile phase.

Table I. Precision and Accuracy of the Method for the Determination of Penciclovir in Human Plasma (*n* = 18)

Concentration (µg/mL)		Relative	Intra-day	Inter-day
Added	Found	error (%)	RSD (%)	RSD (%)
0.10	0.09 ± 0.01	–8.3	4.1	7.0
1.00	0.94 ± 0.03	–6.4	2.4	6.7
5.00	4.86 ± 0.17	–2.8	2.6	6.8

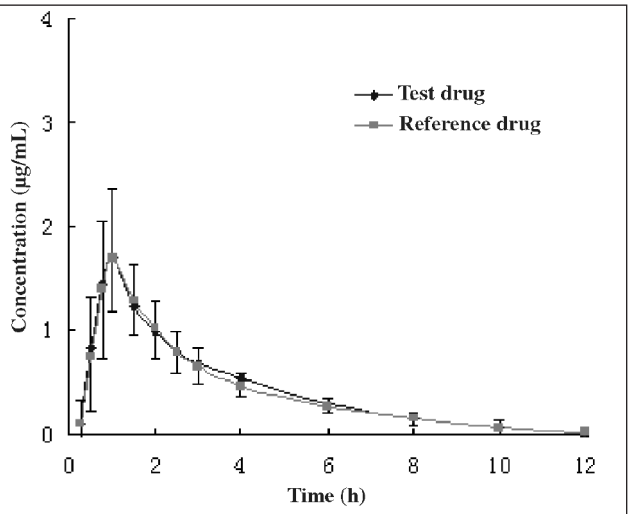

Figure 3. Mean plasma concentration-time profile of penciclovir in 20 volunteers after administration of 250 mg of the two famciclovir capsules.

Separation

Penciclovir and IS were well resolved with respective retention times of 12.0 min and 7.5 min. Compared to penciclovir, the polarity of acyclovir is larger. In a reversed-phase chromatography column, the retention time of acyclovir was shorter than that of penciclovir. Resolution (R) and height equivalence to a theoretical plate for penciclovir or IS are 2.3, 0.020 mm and 1.8, 0.025 mm. The peaks had good shapes and were separated completely. No endogenous interfering peaks were observed at the retention times of penciclovir and acyclovir. Representative chromatograms for blank plasma and blank plasma spiked with penciclovir (2.5 µg/mL) and acyclovir (2.44 µg/mL) are shown in Figures 2A and 2B, respectively. Figure 2C shows the chromatogram of a plasma sample obtained at 0.75 h after a single oral dose of 250 mg famciclovir from a healthy volunteer.

Assay performance

Assay performance of the present method was assessed by the following criteria: linearity, LOQ, recovery, precision, accuracy, and stability.

The standard calibration curves were linear over a concentration range of 0.1–5.0 µg/mL with a mean correlation coefficient of 0.9996. The mean (± standard deviation) regression equation from replicate calibration curves on different days was:

$$Y = (0.4545 \pm 0.03123)X - (0.0083 \pm 0.0007) \quad \text{Eq. 1}$$

where Y is the peak area ratio of penciclovir to the IS, and X is the plasma concentration of penciclovir. The LOQ corresponding with relative standard deviation (RSD) less than 7.0% was 0.1 µg/mL.

The extraction efficiency was determined for three concentrations of QC samples as well as for IS. The mean

extraction recoveries of penciclovir at concentrations of 0.1, 1.0, and 5.0 µg/mL were 96.2 ± 4.4, 96.6 ± 1.5, and 92.1 ± 2.0%, respectively. The extraction recovery of the IS was 98.0 ± 2.5%.

The precision and accuracy were investigated with QC samples at concentrations of 0.1, 1.0, and 5.0 µg/mL. The results are shown in Table I. The intra-day and inter-day precisions of the QC samples were satisfactory with RSD less than 4.1% and 7.0%, respectively. The determined values deviated from the nominal concentration with a relative error (RE) less than –8.3%.

The stability of penciclovir under different conditions was evaluated. The acceptance criteria for all stability tests were at ± 15% of the nominal value. Treated plasma samples were found to be stable for at least 12 h with RE less than –11.0% when the samples were kept at room temperature. The concentrations of penciclovir in plasma, which underwent three freeze-thaw cycles or storage at –20°C for 30 days, were shown to be stable with RE between –5.3% and 4.9%. During the stability study, a standard calibration curve was prepared on each analytical batch.

Bioequivalence study

This method was used for analysis of the drug in human plasma following a single dose oral administration of 250 mg of two famciclovir capsules to twenty healthy volunteers. After a one-week wash-out period, the subjects were crossed-over. Blood samples (3 mL) were withdrawn from the forearm at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, and 12 h after administration, and transferred to tubes containing heparin. The mean plasma concentrations versus time curves of two preparations are shown in Figure 3. C_{max} of 1.86 ± 0.55 µg/mL (reference) and 1.88 ± 0.50 µg/mL (test) were reached at 0.96 ± 0.22 h and 0.89 ± 0.13 h after administration, respectively. AUC_{0-t} and $AUC_{0-\infty}$ for the reference and test drugs were 4.8 ± 1.1, 5.0 ± 1.2 µg/h/mL and 5.3 ± 1.2, 5.5 ± 1.3 µg/h/mL, respectively. Both the mean values and standard deviation were found to be very close between the two preparations. Pharmacokinetic parameters of the two preparations obtained from the statistical calculation exhibited bioequivalent.

Conclusion

The proposed HPLC method was simple, specific, and accurate for the determination of penciclovir in human plasma after oral administration of 250 mg famciclovir. Because of its specificity, good reproducibility, accuracy, inexpensiveness, relative rapidity, and sufficient sensitivity, the method was successfully used in a bioequivalent study. Based on the pharmacokinetic and statistical results, it can be concluded that the two famciclovir capsules were bioequivalent in humans.

References

1. M.R. Boyd, T.H. Bacon, D. Sutton, and M. Cole. Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxy-methlbut-1-y1) guanine (BRL39123) in cell culture. *Antimicrob. Agents Chemother.* **31:** 1238 (1987).
2. M.R. Boyd, T.H. Bacon, and D. Sutton. Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine (BRL 39123) in animals. *Antimicrob. Agents Chemother.* **32(3):** 358 (1988).
3. M.R. Boyd, T.H. Bacon, D. Sutton, and M. Cole. Antiherpesvirus activity of 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine (BRL 39123) in cell culture. *Antimicrob. Agents Chemother.* **31(8):** 1238–42 (1987).
4. R.A. Hodge and R.M. Perkins. Mode of action of 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine (BRL 39123) against herpes simplex virus in MRC-5 cells. *Antimicrob. Agents Chemother.* **33(2):** 223–29 (1989).
5. D. Sutton and M.R. Boyd. Comparative activity of penciclovir and acyclovir in mice infected intraperitoneally with herpes simplex virus type 1 SC16. *Antimicrob. Agents Chemother.* **37:** 642–45 (1993).
6. B.E. Korba and M.R. Boyd. Penciclovir is a selective inhibitor of hepatitis B virus replication in cultured human hepatoblastoma cells. *Antimicrob. Agents Chemother.* **40:** 1282–84 (1996).
7. M.R. Boyd, R. Boon, S.E. Fowles, K. Pagano, D. Sutton, R.A. Vere Hodge, and B.D. Zussman. Some biological properties of BRL 42810, a well absorbed prodrug of the antiherpes virus agent BRL 39123. *Antiviral Res.* **9:** 146 (1988).
8. M.R. Harnden, R.L. Jarvest, M.R. Boyd, D. Sutton, and R.A. Vere Hodge. Prodrugs of the selective antiherpesvirus agent 9-[4-hydroxy-3-(hydroxymethyl)but-1-yllguanine (BRL 39123) with improved absorption properties. *J. Med. Chem.* **32:** 1738–43 (1989).
9. R.A. Vere Hodge, D. Sutton, M.R. Boyd, M.R. Harnden, and R.L. Jarvest. Selection of an oral prodrug (BRL 42810; famciclovir) for the antiherpesvirus agent BRL 39123 [9-(4-hydroxy-3-hydroxymethylbut-l-yl)guanine; penciclovir]. *Antimicrob. Agents Chemother.* **33:** 1765 (1989).
10. M.A. Pue and L.Z. Benet. Pharmacokinetics of famciclovir in man. *Antiviral Chem. Chemother.* **4:** 47–55 (1993).
11. C.W. Filer, G.D. Allen, G.D. Brown, S.E. Fowles, F.J. Hollis, E.E. Mort, and W.T. Prince. Metabolic and pharmacokinetic studies following oral administration of 14C-famciclovir to healthy subjects. *Xenobiotica* **24:** 357 (1994).
12. D.K. Kim, N. Lee, D.H. Ryu, Y.W. Kim, J.S. Kim, K. Chang, G.J. Im, W.S. Choi, Y.B. Cho, K.H. Kim, D. Colledge, and S. Locarnini. Synthesis and evaluation of 2-amino-9-(3-acyloxymethyl-4-alkoxycarbonyloxybut-1-yl) purines and 2-amino-9-(3-alkoxy-carbonyloxymethyl-4-alkoxycarbonyloxybut-1-yl) purines as potential prodrugs of penciclovir. *Bioorg. Med. Chem.* **7:** 1715–25 (1999).
13. S.E. Fowles and D.M. Pierce. High-performance liquid chromatographic method for the determination of 9-(4-hydroxy-3-hydroxymethylbut-1yl) guanine (BRL-39123) in human plasma and urine. *Analyst* **114(11):** 1373–75 (1989).
14. M.A. Pue, S.K. Pratt, A.J. Fairless, S. Fowles, J. Laroche, P. Georgiou, and W. Prince. Linear pharmacokinetics of penciclovir following administration of single oral doses of famciclovir 125, 250, 500 and 750 mg to healthy volunteers. *J. Antimicrob. Chemother.* **33:** 119–127 (1994).
15. F. Schenkel, S. Rudaz, Y. Daali, M. Kondo Oestreicher, J.L. Veuthey, and P. Dayer. Development and validation of a new reversed-phase ion pairing liquid chromatographic method with fluorescence detection for penciclovir analysis in plasma and aqueous humor. *J. Chromatogr. B* **826:** 1–7 (2005).
16. L.C. Hsu, D. J.C. Constable, D.R. Orvos, and R.E. Hannah. Comparison of high-performance liquid chromatography and capillary zone electrophoresis in penciclovir biodegradation kinetic studies. *J. Chromatogr. B* **669:** 85–92 (1995).
17. H.W. Lee, J.H. Seo, and K.T. Lee. 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. *J. Chromatogr. B* **863(1):** 158–62 (2007).

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