Research in Pharmaceutical Sciences, April 2009; 4(1): 47-54
Received: Nov 2008
Accepted: Mar 2009

School of Pharmacy & Pharmaceutical Sciences
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Original Article

HPLC determination of acyclovir in human serum and its application in bioavailability studies

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Abstract

A sensitive, accurate and rapid reverse phase HPLC method was described to quantitate levels of acyclovir in human plasma. The drug, internal standard (metronidazole) and phosphate buffer (0.05 M) were added to serum samples and vortexed for 30 sec. A mixture of isopropyl alcohol:dichloromethane (60:40) was then added and vortexed for 3 min. Samples were centrifuged and the supernatant layer was separated, evaporated to dryness under nitrogen gas stream, reconstituted in mobile phase and, an aliquot of 50 µl was analyzed on a µ-bondapack C_{18} (250 × 3.9 mm) column, with 3% acetonitrile in deionised water and 0.5% orthophosphoric acid, (pH 2.5) at 254 nm. The standard curve covering 100-1500 ng/ml concentration range, was linear, relative errors were within 0.79 to 17.4% and the CV% ranged from 3.81 to 18.2. The limits of quantitation and detection of the method were 100 ng/ml and 25 ng/ml, respectively. The method was suitable for bioavailability and pharmacokinetic studies of acyclovir in humans and applied in a randomized, two-way cross over bioequivalence study of two different acyclovir preparations with twelve subjects and with a one-week washout period.

Keywords: Acyclovir; HPLC; Pharmacokinetics

INTRODUCTION

Acyclovir, 9-[(2-hydroxyethoxy)-methyl]-guanosine, is an acyclic guanosine derivative which exhibits a selective inhibition of herpes viruses replication with potent clinical antiviral activity against the herpes simplex and varicella-zoster viruses (1,2). Bioavailability issues have been of increasing concern to drug regulatory authorities once assessing the safety and efficacy of drug products. Local drug regulatory authorities have, therefore, issued guidelines to ensure adequate bioavailability studies in new drug applications for synonym drugs. A simple and suitable high performance liquid chromatography (HPLC) method for determination of acyclovir levels in serum is of prime need for studying the pharmacokinetics and relative bioavailability of brands of acyclovir preparations.

Immunological techniques (3-5) and HPLC (6-22) are the most commonly used techniques for determination of acyclovir in biological samples. Although immunological techniques have more sensitivity but the entire procedure is time consuming and requires expensive antiserum or monoclonal antibodies which are not cost-benefit (18).

Several HPLC methods have been reported for determination of acyclovir in human serum using UV (6-17) or fluorescence detection (18-22). Because acyclovir is soluble in aqueous medium and practically insoluble in most organic solvents, protein precipitation (PP) with perchloric acid (6,7,10,12,16,18,19,20,22) or solid phase extraction (SPE) (8,9,11,14,21,23) have been utilized to clean up the serum samples. However, these sample pretreatment procedures suffer from some disadvantages.

PP can deteriorate the chromatographic column even with low volume of injection due to either high acidity or inadequate precipitation of the protein contents in the samples. This reduces the number of samples which can

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be analyzed. Sample dilution which occurs after deproteinization also reduces the sensitivity of the assay. In some reports 35% (22) to 62% (20) perchloroic acid has been utilized to precipitate the plasma protein which can deteriorate the analytical column and harm the injection port in long run. Injection of the acid supernatant also leads to numerous late-eluting peaks that time consuming gradient elution is needed to remove them.

SPE technique is a fairly expensive procedure and suffers from sorbent drying between washing intervals which may result in cracking of the packing material. Due to the binding of drug to the sorbent, nonpolar solvent will not be efficient to elute the drug. Therefore, sample dilution with aqueous extractant occurs and as these solvents can not be easily evaporated, a decrease in sensitivity of the assay is unavoidable. To overcome the lower sensitivity associated with SPE and PP cleanup procedures, fluorescence detection should be utilized which is not readily available in most laboratories due to the financial reasons and also extremely pure HPLC grade solvents are required. In most of the above mentioned reports, internal standard has not been utilized (6-11,16,17,20,21-25) which would result in more variations and consequently lower sensitivity. In the light of above considerations, it seems that development, validation, and application of a HPLC method for determination of acyclovir in plasma based on liquid-liquid extraction (LLE), UV detection and appropriate internal standard is imperative. An extensive literature review revealed only a single report of acyclovir HPLC assay in biological samples using LLE as a clean up procedure (15). Therefore, the aim of the present study was twofold. Firstly to describe a simple, rapid, sensitive, reliable, and cost-effective HPLC method utilizing LLE and UV detection for determination of acyclovir in human serum and secondly, to apply the validated method to a bioequivalence study of this drug on a domestic formulation.

MATERIALS AND METHODS

Reagents and solutions

Acyclovir was supplied by Farabi pharmaceutical Co. (Iran). Internal standard, metronidazole, was from Amin Pharmaceutical Co. (Iran). HPLC grade acetonitrile, isopropyl alcohol and dichloromethane were purchased from Caledon (Canada). KH_2PO_4, triethylamine and orthophosphoric acid were from Merck (Germany). All other chemicals were of analytical grades. Water was obtained by double distillation and purified additionally with a Milli-Q system. Brands and reference (Acyclostad®) of oral tablets containing 400 mg acyclovir were supplied by Farabi Pharmaceutical Co. (Iran) and Stada (Germany), respectively.

Instrumentation

A Younglin SP930D HPLC pump, UV/730D absorbance detector, chromatopac integrator, and a Rheodyne injector was employed to carry out the acyclovir assay.

Chromatographic conditions

A C_{18} µ-bondapack (250 × 3.9 mm, Waters, Ireland) was used as chromatographic column. The mobile phase consisted of KH_2PO_4 (0.02 M) in double distilled deionized water: acetonitrile (97:3). Twenty µl of triethylamine and adequate orthophosphoric acid were added to obtain final pH of 2.5 ± 0.1. The aqueous phase was eluted at a flow rate of 1.6 ml/min and effluent was monitored at 254 nm. Quantitation was achieved by measurement of the peak area ratios of the drug to the internal standard.

Standard solutions

A stock solution of acyclovir was prepared by dissolving 20 mg of acyclovir in 100 ml of mobile phase in a 100 ml volumetric flask to obtain the concentration of 200 µg/ml. A series of standard solutions at concentrations of 2, 4, 10, 15, 20, 30 µg/ml were prepared by dilution of the stock-solution in mobile phase to obtain different working solutions. The stock and working solutions were prepared in the morning of each day freshly. Stock solution of metronidazole at concentration of 50 µg/ml was prepared in methanol and stored at 4 ºC.

Sample preparation

To 1 ml of plasma in 10 ml test tubes containing 50 µl of internal standard solution, 100 µl of phosphate buffer (0.05 M) was added and then the tubes were vortexed for 30 s. For extraction, 6 ml of the mixture of isopropyl alcohol:dichloromethane (60:40) was added and samples were vortexed for 3 min and subsequently centrifuged at 3000 rpm for 15 min.

The upper layers were then transferred to clean test tubes and evaporated under the nitrogen gas. The residues were reconstituted in 100 µl mobile phase, vortexed for 30 s and centrifuged at 13000 rpm. A 50 µl volume of the final clear solution was then injected in to the HPLC system.

Selectivity and specificity

The presence of possible disturbing endogenous peaks was examined on control human plasma samples obtained from twelve healthy volunteers. These samples were pre-treated according to the sample preparation procedure except for the addition of internal standard.

Recovery determination

The extraction recoveries of acyclovir at the concentration range of calibration curve were evaluated by dividing the peak areas obtained after extraction of known amount of acyclovir from plasma to those obtained from the same amounts of unextracted acyclovir.

Precision and accuracy

The intra- and inter-day variations of the assay were determined by replicate analysis (n=5) of calibration samples of acyclovir at concentrations within the range of calibration curve (100-1500 ng /ml) in a single analytical run on the same day and in five different days, respectively, using fresh stock solutions and plasma batches. The percent relative standard deviations of results of the assay were determined.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The parameter LOD was determined using the signal-to-noise ratio by comparing results of the test of samples with known concentrations of analyte to blank samples. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. The LOQ was identified as the lowest plasma concentration of the standard curve that could be quantified with acceptable accuracy, precision and variability.

Calibration curves and quantitation

Blank plasma was prepared from heparinized whole-blood samples collected from healthy volunteers and stored at -20 °C. Calibration samples of acyclovir were prepared in plasma. To separate tubes, 1 ml of blank plasma, 100 µl of phosphate buffer (0.05 m), 50 µl of internal standard at fixed concentration of 50 µg/ml and 50 µl of acyclovir standard solutions at concentrations of 2, 4, 10, 15, 20 and 30 µg/ml were added to yield standard acyclovir solutions at concentrations of 100-1500 ng/ml. The samples were taken through the preparation procedure described above and an aliquot of 50 µl of the final solution was injected in to the column. Calibration curves were constructed by plotting peak area ratio (y) of acyclovir to the internal standard versus acyclovir concentrations (x). Linear regression was used to quantitate plasma drug concentrations in samples through determination of the peak area of acyclovir to internal standard and comparison of the resulting values with those of the standard curve which was obtained after analysis of calibration samples.

Application of the method

The present method was applied to a comparative bioavailability study. The ethics committee on human studies of the Isfahan University of Medical Sciences approved the study. Twelve healthy adult male volunteers aged between 21 to 25 years and weighing from 60 to 85 kg participated in the study. Based on laboratory tests including hematology, blood biochemistry and urine analyses, subjects did not have an evidence of hepatic, renal, gastrointestinal or hematological deviations. The subjects were instructed to abstain from taking any medication at least 2 weeks prior to and during the study period. Informed

consent was obtained from the subjects after explaining the nature and purpose of the study. The protocol was the conventional, two-way, crossover study with twelve subjects and a one-week washout period. In the first trial period, after an overnight fasting, subjects were given a single oral dose of 400 mg acyclovir of either test (Farabi Pharmaceutical Company) or reference (Acyclostad®, Stada) products in a randomized fashion with 200 ml of water. The drugs were administrated after an overnight fasting and blood sampling was carried out at 0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h after drug administration. The blood samples were centrifuged at 3500 rpm for 20 min; serum was separated and kept frozen at -20 ºC in coded glass tubes.

Pharmacokinetic analysis

Acyclovir pharmacokinetic parameters were determined by non-compartmental method. The elimination rate constant (k_e) was obtained from the least square fitted terminal log-linear portion of the plasma concentration-time profile. The area under the curve to the last measurable plasma concentration (AUC_{0-t}) was estimated by the linear trapezoidal rule. The area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by equation of $AUC_{0-t} + C_t / k_e$ where C_t is the last measurable concentration. The peak plasma concentration (C_{max}) and time to peak concentration (T_{max}) were determined by inspection of the individual drug plasma concentration-time profiles.

Statistical analysis

For the purpose of bioequivalence analysis, AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} were considered as primary variables. A difference between two related parameters was considered statistically significant for a *P*-value equal to or less than 0.05. The 90% confidence intervals of the mean pharmacokinetic parameters and paired student's t-test were estimated. All statistical analyses were performed using SPSS 10. In addition, Wilcoxon test was applied to compare T_{max}. Statistical significance was set at 0.05.

RESULTS

Specificity and selectivity

The chromatograms of human blank plasma and human plasma spiked with acyclovir and the internal standard are shown in Fig. 1.

Recovery

The extraction efficiency was determined by dividing of the peak areas of the spiked plasma samples to those of un-extracted acyclovir solution. The recoveries were consistent for the samples as demonstrated by coefficient of variation (CV) of 7.45%. Recoveries were not dependent on the concentration, which consequently resulted in good linearity of the calibration curve. The mean recoveries were found to be 91.7% which was comparable to the previous single report using LLE as extraction and clean up procedures (15).

Accuracy and precision

The results of within- and between-day variability are presented in Table 1. The CV% ranged from 3.8 to 18.2% and relative errors were within 0.79 to 17.4%. Results of coefficients of variations and percent errors which are below 20% indicate that method is reproducible within day and between days (26).

Linearity, limit of detection and limit of quantitation

The standard curves were linear over the concentration ranges of 100-1500 ng/ml using line-fit plot in regression analysis with a coefficient of 0.998.

The detection limit for acyclovir was approximately 25 ng/ml at a signal to noise ratio of 3:1 and the limit of quantitation was 100 ng/ml which was comparable or to some extent higher than some of the previously reported methods using UV or fluorescence detectors (6,12,17,18,25,27). Acyclovir was measurable at the first sampling time (0.25 h) and after 4 half-lives in all volunteers. The use of internal standard increases the accuracy of the assay whose availability is an important issue in HPLC assays.

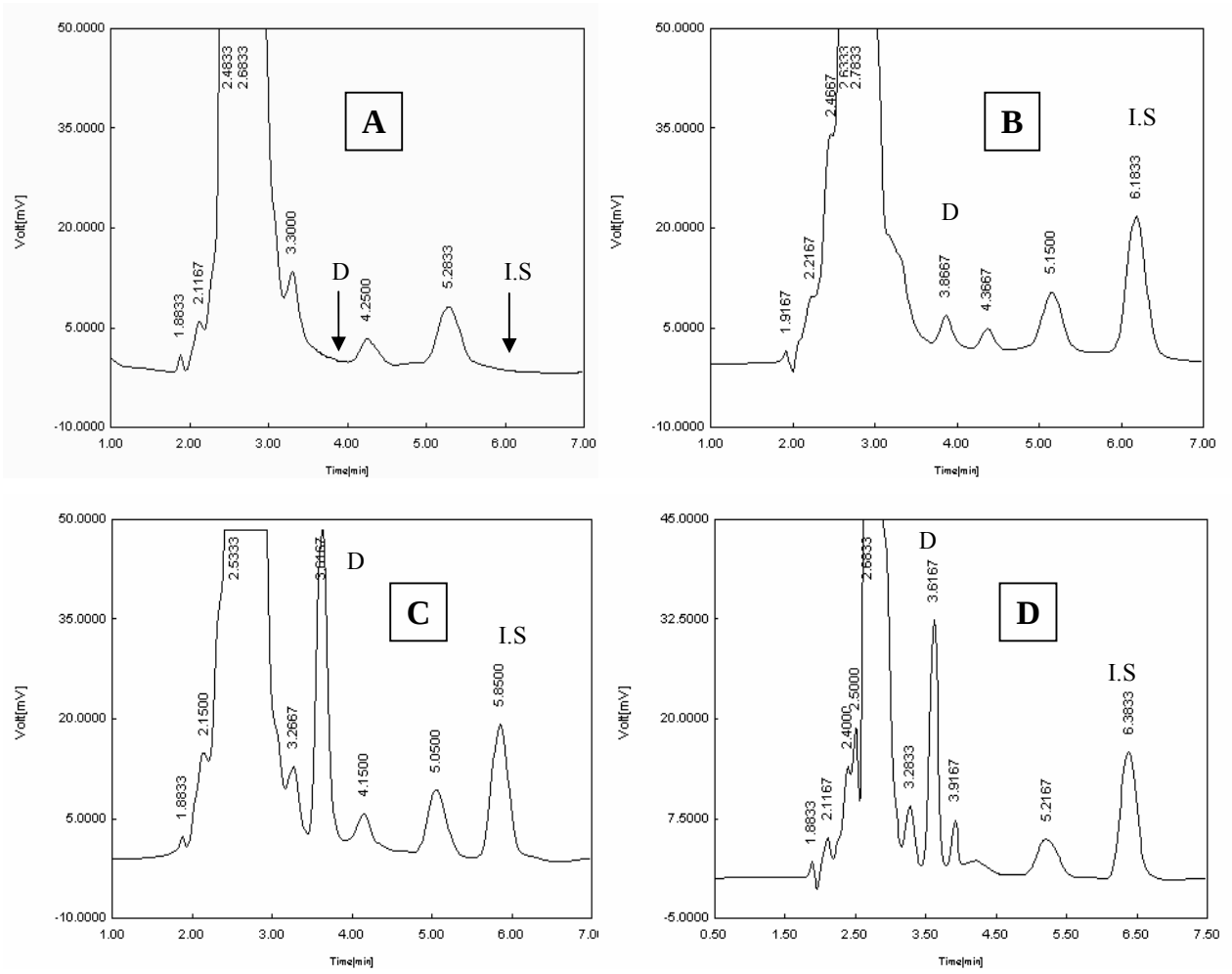

Fig. 1. Chromatograms of blank human plasma A) blank plasma spiked with internal standard and drug at concentration of 100 ng/ml, B) blank plasma spiked with internal standard and drug at concentration of 1500 ng/ml and C) plasma sample obtained at 1.25 h after a single oral dose of 400 mg acyclovir from a healthy volunteer containing 913 ng/ml of acyclovir (D). (D stands for Drug and IS for internal standard).

Table 1. Intraday and interdays variability of the HPLC assay for determination of acyclovir concentration in plasma.

C (ng/ml)	Intraday variability				Interday variability			
	Mean (ng/ml)	SD	CV%	Error%	Mean(ng/ml)	SD	CV%	Error%
100	96	18.3	17.1	17.40	86	18.9	18.2	13.9
200	187	7.0	3.8	3.70	182	24.7	13.6	2.3
500	527	48.7	9.2	2.42	515	49.3	9.6	11.7
750	746	82.3	11.0	0.79	758	74.3	9.8	12.2
1000	1141	115.2	10.1	4.29	1015	130.1	12.8	9.7
1500	1502	77.8	5.2	5.23	1463	114.4	7.8	9.9

Application of the method

This method was applied to the determination of acyclovir in serum following single oral administration of 400 mg of two different acyclovir preparation, namely Acyclostad® (Stada) and a generic equivalent (Acyclavir, Farabi) in 12 healthy volunteers. A typical serum concentration-time profile for both formulations is depicted in Fig. 2 and resulted pharmacokinetic parameters are shown in Table 2.

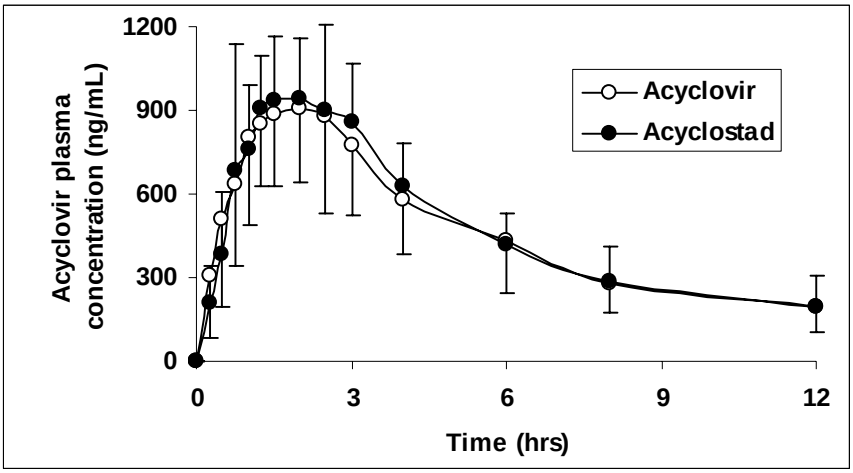

Fig. 2. The mean plasma acyclovir levels vs. time profiles in 12 healthy volunteers following single oral dose of 400 mg of either test or reference preparations.

Table 2. Pharmacokinetic parameters following administration of 400 mg acyclovir to 12 healthy volunteers.

Parameters	Test	Reference	p-value	90% CI[a]	Wilcoxon
T_{max} (h)	2.08 ± 0.61	1.94 ± 0.59	0.487	NA[b]	0.502
C_{max} (ng/ml)	1069 ± 245	1167 ± 300	0.337	0.844 - 1.08	NA
AUC_{0-12} (ngh/ml)	5556 ± 1301	5684 ± 1010	0.736	0.868 - 1.112	NA
$AUC_{0-\infty}$ (ngh/ml)	6907 ± 1812	6972 ± 1727	0.888	0.891 - 1.134	NA

[a]Confidence Interval; [b]Not Applicable

DISCUSSION

Several HPLC methods using PP or SPE have been reported for measurement of acyclovir in biological samples (6-22). Either clean-up procedure results in sample dilution and consequently low sensitivity. The detection limit have been improved by increasing injection volume and/or utilization of highly acidic mobile phase because the fluorescence of acyclovir increases dramatically with increasing acidity of the mobile phase, especially below pH 2 (27). However, increasing injection volume leads to a significant reduction in the lifetime of the analytical column; numerous late running peaks, high background noise, and broaden peaks (15,18). Strongly acidic mobile phase lead to the rapid deterioration of the analytical column and increased retention time and only about 600 samples could be analyzed without deterioration of the column (22). Neutralization of the strongly acidic supernatant by phosphate buffer could possibly be used for acyclovir (28), however, this lengthens the process and necessitates further separation of potassium or sodium percholorate which consequently reduces the sensitivity. In this study, perchloric acid, acetonitrile, methanol, and zinc sulfate solutions were used to precipitate the serum proteins but the sensitivity did not reach to the extent which was appropriate for pharmacokinetic studies. PP which was followed by dichloromethane or ethyl acetate extraction was not efficient enough in extracting acyclovir from serum samples. Therefore, LLE seemed more appropriate extracting procedure for acyclovir compared with PP and SPE.

In the present study a reproducible and sensitive HPLC assay for acyclovir analysis in human serum that can be used for pharmacokinetic studies in humans using LLE was described. No endogenous peaks from serum were found to interfere with the elution of the drug or internal standard. Acyclovir and

the internal standard were well resolved with good symmetry with respective retention times of 3.8 and 6.1 min. No interfering peaks were observed in the chromatogram of blank human plasma or the chromatograms of blank plasma from 12 healthy individuals who participated in the study. In the present assay neither highly acidic mobile phase, nor high injection volume of acidic supernatant was used. This protects the column from early deterioration and therefore numerous samples could be analyzed without exposing to any aforementioned drawbacks.

Because acyclovir is soluble in aqueous solutions, fairly polar extracting solvents should be applied to extract this agent from biological fluids. In addition to the previously reported extracting solvents (15) we tested various combination of heptane/isoamyl alcohol as extracting solvents, but they were not efficient enough in extracting the drug from serum specimen. Therefore, finally the mixture of dichloromethane:isopropyl alcohol at ratio of 40:60 v/v which was slightly different from what (50:50 v/v) has been reported previously (15) was applied. Due to the lower density of isopropyl alcohol (0.79 g/ml) compared to dichloromethane (1.33 g/ml), the density of 40:60 ratio used in this study is smaller than unity. This rendered the extracting solvent to float on top of serum sample and easily could be withdrawn.

In most reported HPLC methods for analysis of acyclovir an internal standard was not used (6-11,16,17,20,21-25) which resulted in reduced precision and reproducibility of the assay. In some previous reports guanosine which is structurally related to acyclovir was used as internal standard (18). In sensitive assays, however, this may interfere with endogenous guanosine.

In the present study vanillin which was already reported (15) was used as the internal standard but appeared as a very late eluting peak. Therefore, few other chemicals including phenacetine, ranitidine, and metronidazole with similar UV spectrum and solubility properties to acyclovir were tested as potential internal standard. Phenacetine with retention time of 3.5 min interfered with serum endogenous peaks. Metronidazole and ranitidine eluted at 8.2 and 11 min, respectively. As a result metronidazole was selected as the internal standard considering its UV spectrum, shorter retention time, and the distance from acyclovir peak, structural similarity, and interference with endogenous peaks. In this study less acidic mobile phase was used as compared with previous report using LLE procedure (15). This may elongate the life time of HPLC column. In contrast to other reports (15), the mobile phase applied in the current study did not contain sodium dodecyl sulfate or strong organic alkaline.

The parameters t_{max} and $AUC_{0-\infty}$ corresponds to the respective rate and extent of drug absorption, while C_{max} is related to both of these two processes, with all three measures being essential for comparison of the bioavailability of the two preparations. The AUCs, T_{max}, and C_{max} for the two products were not statistically different ($P> 0.05$), suggesting that the serum profiles generated by Acyclostad® were comparable to those produced by acyclovir manufactured by Farabi company. Ninety percent confidence intervals of the AUCs and C_{max} were also found to be within the FDA acceptable range of 80-120% for bioequivalance evaluation indicating no statistically difference between generic formulation and reference.

In conclusion, good precision, accuracy, simplicity, reproducibility, shorter time of analysis and sufficient sensitivity of the method makes it particularly useful for processing of multiple samples in a limited period of time for pharmacokinetic studies of acyclovir.

ACKNOWLEDGMENT

The authors are grateful to Farabi Pharmaceutical Company (Isfahan, Iran) for the financial support ot this work.

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