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Short communication

Determination of acyclovir in human serum by high-performance liquid chromatography using liquid–liquid extraction and its application in pharmacokinetic studies

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Abstract

A fast, simple and sensitive high performance liquid chromatographic (HPLC) method has been described for determination of acyclovir in human serum. Since acyclovir is a polar compound and soluble in aqueous medium and practically insoluble in most of organic solvents, its analysis in biological fluids in currently published HPLC methods, involve pre-treatment of acyclovir plasma sample including deproteinization or solid phase extraction. In present method liquid–liquid extraction of acyclovir and internal standard (vanillin) is achieved using dichloromethane-isopropyl alcohol (1:1, v/v) as an extracting solvent. Analysis was carried out on ODS column using methanol-phosphate buffer (0.05 M) containing sodium dodecyl sulfate (200 mg/L) and triethylamine (2 mL/L, v/v) as mobile phase (pH = 2.3; 5:95, v/v) at flow rate of 2 ml/min. The method was shown to be selective and linear into the concentration range of 10–2560 ng/mL. Accuracy and precision of the method were also studied. The limit of quantitation was evaluated to be 10 ng/mL. This method was applied in bioequivalence study of two different acyclovir preparations after administration of 400 mg in 12 healthy volunteers.
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Keywords: Reverse phase chromatography; HPLC; Acyclovir; Serum; Bioequivalence study

1. Introduction

Acyclovir, 9-[(2-hydroxyethoxy)-methyl]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]. As acyclovir is structurally similar to endogenous substances, its analysis in human serum is complicated and requires high selective analytical methods. Immunological techniques [3–5] and HPLC are the most common used methods for determination of acyclovir in biological samples. Radioimmunoassay methods are sensitive but require antiserum or monoclonal antibodies development and are expensive. Several HPLC methods [6–21] have been published for determination of acyclovir in human serum using UV or fluorescence detection. Since acyclovir is a polar compound and soluble in aqueous medium and practically insoluble in most of organic solvents, protein precipitation with perchloric acid [6–8,12,16,17] or solid phase extraction [9–11,14,15,20] are applied for pre-treatment of the drug in serum samples. While the sensitivity of analysis is significantly reduced due to dilution of the samples after deproteinization, injection of the acid supernatant after precipitation of proteins by perchloric acid leads to numerous late-eluting peaks and significant reduction of the lifetime of analytical column. Time consuming gradient elution is needed for removing of the late-eluting peaks, and deterioration of column performance significantly reduces the number of samples which can be analyzed. Solid phase extraction is expensive and moreover about 1 ml of solvent is required for elution of the drug from the cartridges. How-

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ever, in solid phase extraction methods, the drug is eluted by application of aqueous solvents. As these solvents cannot easily be evaporated, dilution of the samples reduces the method sensitivity. Different limits of quantification (LOQ) ranging from 10 to 200 ng/mL of serum have been reported in published methods, however; LOQ of less than 50 ng/mL has been achieved in these methods by either increasing the injection volume [6,8,9,11,13] and/or application of highly acidic mobile phase [12] and at the expense of rapid deterioration of the analytical column. Limit of quantification of 10 ng/mL has been obtained in method described by Swart et al. [9] using specific polymeric Oasis^{TM} extraction column and injection volume of 130 μl. However, the corresponding recovery of acyclovir in this method in which calibration curves were linear between the ranges of 5–1200 ng/mL was less than 50%. Their extraction procedure needs to expensive specific polymeric Oasis^{TM} extraction column and is more time consuming than the deproteinization technique. A protein precipitation method (8) with LOQ of 20 ng/mL and injection volume of 100 μl has also been reported. However, this method involves long retention time of acyclovir and insufficient sensitivity for analysis of the drug up to 24 h following single dose bioequivalence studies.

Present paper describes a simple, economic and yet sensitive HPLC method for determination of acyclovir in human serum using liquid–liquid extraction. This method in which the LOQ has been improved without the using of highly acidic mobile phase or large injection volume has been applied in a bioequivalence study.

2. Experimental

2.1. Reagent and chemicals

Acyclovir was from Sigma (Sigma, St. Louis, MO, USA) and kindly provided by Bakhtar Bioshimi pharmaceutical company (Kermanshah, Iran). HPLC-grade methanol, monobasic sodium phosphate, phosphoric acid, triethylamine, sodium dodecyl sulfate, vanillin (I.S.), 2-propanol and dichloromethane purchased from Merck (Darmstadt, Germany). Water was glass-double distilled and further purified for HPLC with a maxima purification system (USF ELGA, England).

2.2. Preparation of standard solutions

Stock solutions of acyclovir (200 μg/ml) and vanillin (25 μg/ml) were prepared by dissolving the drug in deionized water and stored in a refrigerator at 4 °C. It remained stable for at least one month. Acyclovir stock solution was further diluted with methanol to obtain the different working solutions ranging from 10 ng/mL to 2560 ng/mL.

For preparation of serum calibration curves samples, 100 μl each of working standard acyclovir solutions within the concentration range of 10–2560 ng/ml were evaporated in glass tubes (16 mm × 100 mm) under gentle stream of nitrogen at 50 °C, after addition of 1 ml human blank serum and mixing for 10 s on a vortex mixer, the samples were subjected to extraction and analysis.

2.3. Apparatus and chromatographic condition

The chromatographic analysis performed on a LC-10A (Shimadzu, Kyoto, Japan). System consisted of two pumps (LC-10A), a system controller (SCL 10AD), a UV–VIS spectrophotometric detector operated at 250 nm (SPD-10A), and rheodyne injection valve with a 20 μl filling loop, a column oven (CTO-10A) set at 62 °C, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. The analysis was performed on a reverse phase column (150 mm × 6 mm i.d.) which was packed with 5 μm particles of ODS packing material (Shimpack-CLC-ODS). A guard column (4.0 mm i.d. × 1 cm; Shim-pack G-ODS, Shimadzu Japan) packed with the same packing material was placed before the inlet of the analytical column. The mixture of methanol-phosphate buffer (0.05 M) containing sodium dodecyl sulfate (200 mg/L) and triethylamine (2 mL/L, v/v) was used as mobile phase (pH = 2.3; 5:95, v/v). The eluent was filtered through a 0.45 μm filter (Milipore, Bedford, MA, USA), degassed and pumped at flow rate of 2 ml/min.

2.4. Sample preparation

To 1 ml serum samples in glass tubes (16 mm × 100 mm) 100 μl of the I.S. (25 μg/ml) and 5 ml dichloromethane-isopropyl alcohol (1–1, v/v) were added. After mixing for 30 s on a vortex mixer and centrifugation (5 min at 6000 × *g*), the organic phase was removed and evaporated to dryness under stream of nitrogen at 50 °C. The residue was reconstituted with 100 μl of water, syringe filtered using 0.45 μm syringe filter and a volume of 20 μl was injected into the HPLC system.

2.5. Calibration and method validation

Serum samples obtained from healthy volunteers were used for method validation and linearity studies. Calibration curves (unweighted regression line) were obtained by linear least-squares regression analysis plotting of peak-area ratios (acyclovir/I.S.) versus the acyclovir concentrations. The presence of disturbing endogenous peaks was examined on twelve human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. The recoveries of acyclovir at the concentration range of calibration curve as well as the I.S. at applied concentration were calculated by comparing peak areas obtained after extraction of known amounts of acyclovir from serum, with peak areas obtained from the same amounts of unextracted acyclovir. Intra and inter day variations were determined by repeated analysis ($n = 6$) of different acyclovir concentrations within the range

of calibration curve in a single analytical run and in 10 analytical run performed on different days respectively using the same stock solutions and plasma batches. The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 3:1. The limit of quantification was defined as the lowest serum concentration of acyclovir quantified with a coefficient of variation of less than 20%. Stability of solutions of acyclovir and vanillin was studied at the applied concentration over a period of 30 days by comparing of the peak areas at different times. Stability of acyclovir in serum samples was studied by comparing of the determined concentration in different times up to 60 days maintenance of the samples at −40 °C.

3. Application of the method

The present method has been applied in a randomized crossover bioequivalence study in 12 healthy volunteers following single oral administration of 400 mg acyclovir from either Bakhtar Bioshimi (acyclovir) or Wellcome (Zovirax) pharmaceutical companies. The drugs were administrated under fasting conditions and blood sampling were carried out at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8, 10 and 12 h after drug administration. Pharmacokinetic parameters were calculated and compared using paired Student's *t*-test and statistical significance was defined at the level of $P < 0.05$.

4. Results and discussion

4.1. Specificity and selectivity

Chromatograms of human blank serum and human serum spiked with acyclovir (20 ng/mL) and the I.S. are shown in Fig. 1A and B, respectively. No endogenous peaks from serum were found to interfere with the elution of the drug or I.S. Acyclovir and the I.S. were well resolved with good symmetry with respective retention times of 304 and 5.7 min. Fig. 1C shows the chromatogram of serum sample obtained at 3 h after a single oral dose of 400 mg acyclovir from a healthy volunteer.

4.2. Recovery, accuracy and precision

The recoveries of acyclovir and the I.S. from serum were examined by extracting of spiked serum samples comparing with peak areas obtained after the same amounts of unextracted acyclovir solutions in deionized water. The mean recoveries were found to be 90 ± 8% for acyclovir and 95 ± 3% for the I.S. The intra-day and inter-days accuracy and precision values of the assay method are presented in Tables 1 and 2, respectively. The mean inter-days precision was from 12.8% (low concentration) to 1.0% (high concentration) and the intra-day precision from 12.1 to 1.1% determined on spiked samples. The accuracy of the method was 94.4–102.5% (inter-days) and 99.6–106.7% (intra-day).

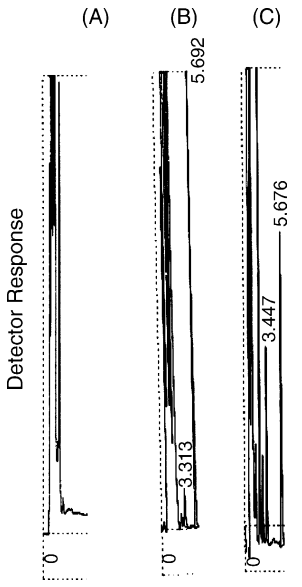

Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum (B) human blank serum spiked with 20 ng/mL acyclovir and the I.S. (full scale is 20 mV) and (C) serum samples obtained at 3 h after a single oral dose of 400 mg acyclovir from a healthy volunteer containing 640 ng/mL of acyclovir (full scale is 40 mV).

Table 1
Intra-day precision and accuracy for determination of acyclovir in human serum by the HPLC method

Concentration added (ng/mL) intra-day	Mean concentration found (ng/mL)	Precision (%)	Accuracy (%)
10	10.7 ± 1.2	12.1	6.7
20	20.1 ± 1.4	7.14	0.7
80	81.5 ± 2.1	2.63	1.9
320	318.7 ± 4.0	1.26	−0.4
640	641.8 ± 1.2	1.18	0.3
2560	2581 ± 27.7	1.08	0.8

4.3. Linearity and stability

The limit of detection was approximately 4 ng/mL at a signal to noise ratio of 3:1 and limit of quantification corre-

Table 2
Inter-day precision and accuracy for determination of acyclovir in human serum by the HPLC method

Concentration added (ng/mL) inter-day	Mean concentration found (ng/mL)	Precision (%)	Accuracy (%)
10	9.4 ± 1.2	12.83	−5.6
20	20.5 ± 1.8	8.72	2.5
80	80.8 ± 1.9	2.4	1.0
320	322 ± 5.9	1.83	0.6
640	646.5 ± 9.4	1.44	1.0
2560	2552.3 ± 26.6	1.04	−0.3

Table 3
Assay linearity for determination of acyclovir in human serum by the HPLC method

	Correlation coefficient of the linear regression analysis[a] ($r \pm$ S.D.)	Slope (b) (mean ± S.D.)	Intercept (a) (mean ± S.D.)
Inter-day reproducibility ($n = 4$)	0.9988 ± 0.0019 (C.V. = 0.2%)	0.09325 ± 0.00457 (C.V. = 4.9%)	2.4663 ± 0.3246 (C.V. = 13.2%)
Intra-day reproducibility ($n = 10$)	0.9942 ± 0.0028 (C.V. = 0.29%)	0.08659 ± 0.0072 (C.V. = 8.1%)	2.4865 ± 0.37799 (C.V. = 15.2%)

r = correlation coefficient.
[a] Linear unweighted regression, formula: $y = bx + a$.

sponding with a coefficient of variation of less than 20% was 10 ng/mL. The standard calibration curves were linear over the concentration ranges of 10–2560 ng/mL using line-fit plot in regression analysis with a coefficient of 0.9988. Intra-day reproducibility was determined for calibration curves prepared the same day in replicate ($n = 4$) using pooled serum sample and the same stock solutions. Inter-day reproducibility was determined for calibration curves prepared on different days ($n = 10$) using pooled serum sample and the same stock solutions. Results are given in Table 3. Stock solutions of acyclovir and vanillin were stable at least for 30 days when stored at 4 °C. Extracted serum was found to be stable for at least 24 h if the samples were kept at room temperature. After 60 days the concentrations of acyclovir in serum stored at −40 °C were found to be 101 ± 3% from the initial values.

4.4. Application of the method and conclusions

This method has been applied to the determination of acyclovir in serum following single oral administration of 400 mg of two different acyclovir preparations, namely Zovirax (Wellcome) and a generic equivalent (Acyclovir, Bakhtar Bioshimi) in 12 healthy volunteers. Fig. 2 shows a typical serum concentration–time profile for both formulations and resulted pharmacokinetic parameters are summarized in Table 4. In our method 1 ml of serum sample has been used and although the sensitivity of was enough for analysis of acyclovir up to 24 h after single dose study of the drug, however, unlike protein precipitation methods the LOQ can be improved by increasing the sampling volume.

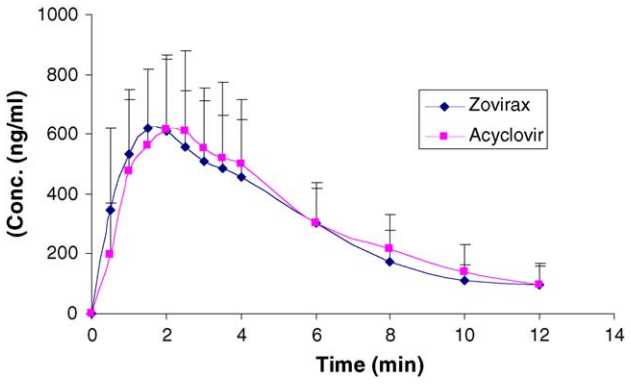

Fig. 2. Mean serum concentrations–time profiles of acyclovir in 12 human volunteers following single oral dose of 400 mg of either test (Bakhtar Bioshimi) or reference (Wellcome) preparations.

Table 4
Mean (S.D.) pharmacokinetic parameters of acyclovir in 12 human volunteers after administration of a single 400 mg oral dose

Parameter/Prep.	Test	Reference	P-value[a]
T_{max} (h)	2.81 (1.0)	2.42 (0.72)	NS
C_{max} (ng/mL)	791.1 (264.2)	801.3 (330.4)	NS
$AUC_{0–72}$ (ng h/ml)	3834.2 (1388.6)	3817.4 (1516.6)	NS
$AUC_{0–\infty}$ (ng h/ml)	4369.4 (1733.1)	4548.7 (1930.2)	NS
$T_{1/2}$ (h)	3.4 (1.1)	3.85 (1.7)	NS

[a] NS = no significant difference ($P < 0.05$).

5. Discussion

Several HPLC methods using protein precipitation or solid phase extraction have been reported for analysis of acyclovir in biological samples, but the assay reported here is the first to report liquid–liquid extraction of acyclovir from human serum. In different published methods improvement of detection limit have been achieved by either increasing the injection volume and/or application of highly acidic mobile phase, but increasing the injection volume leads to reduction the lifetime of analytical column, numerous late-eluting peaks and high background noise, while strongly acidic mobile phase may lead to rapid deterioration of the analytical column. In published deproteinization methods using perchloric acid, the injection by HPLC of the acid supernatant contributes significantly to the reduction the column life time even when the volume injection is low and in any case only 600 samples could be analyzed without deterioration of the column [22]. In our method however, neither strongly acidic mobile phase, nor high injection volume of acidic supernatant have been used. Thus more than 1000 samples were analyzed without deterioration of the performance of the column.

Protein precipitation by perchloric acid and neutralization of the supernatant by phosphate buffer has been reported for analysis of gancyclovir [23] and can be applied for acyclovir however, this is time consuming and needs to further separation of potassium or sodium percholorate and reduces the sensitivity. In this study protein precipitation with either trichloroacetic acid, perchloric acid or acetonitrile, has initially been evaluated, but resulted detection limit was not low enough for pharmacokinetic studies. Direct protein precipitation followed by the extraction of the supernatant with hexane or chloroform gave low recovery. Acyclovir is soluble in aqueous solutions, thus polar solvents are required for its extraction from the serum. Extraction efficiency of different solvents including ethyl acetate, dichloromethane and chloroform each alone and in combination with differ-

ent percents of 2-propanol were tested. Prior to selection of extracting solvent of, the factors affecting the separation containing pH and salting out approach were studied in order to obtain the optimum conditions. Neither pH adjustment nor salting out approach had any important effect on recovery and the maximal yields of recoveries for both acyclovir and the I.S. were obtained using the mixture of dichloromethane-2-propanol (1:1, v/v) without any pH adjustment. Increasing of the dichloromethane ratio in extracting solvents leads to more clean extraction but at the expense of reduction in acyclovir recovery. Thus sufficient amount of 2-propanol in extracting solvent is essential. Using the developed method of extraction, good separation of the drug and I.S. were achieved without endogenous peaks from serum as shown in Fig. 1.

Some potentially co-administered drugs including; zidovudine, lamivudine, nelfinavir, fluconazole, itraconazole, trimethoprim, sulfamethoxazole, ofloxacin, ciprofloxacin, acetaminophen, promethazine, phenytoin and Phenobarbital were checked for interference with acyclovir analysis and other than lamivudine and nelfinavir peaks which appeared at 10 and 11.5 min, respectively, none of these drugs eluted up to 20 min under above conditions.

In most published HPLC methods for acyclovir an internal standard has not been used which result in reducing of precision and reproducibility of the analysis. In our study however, many drugs and chemicals were tested to select I.S. but, application of mobile phase with low percent of organic solution and liquid extraction of the drug with high percent of isopropyl alcohol, result in low recovery and/or inappropriate retention time of most of them. Vanillin was selected as the I.S. considering its UV spectrum, retention time, recovery and interference with the endogenous peaks.

In conclusion the present method is rapid, simple, and reproducible with limit of quantification of 10 ng/mL for 1 ml of serum. In this method neither highly acidic mobile phase nor increasing the injection volume were used for increasing of the method sensitivity.

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