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Rapid, simple and sensitive high-performance liquid chromatographic method for detection and determination of acyclovir in human plasma and its use in bioavailability studies

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Abstract

A rapid, simple and sensitive reversed-phase high-performance liquid chromatographic (HPLC) method has been developed for the measurement of acyclovir concentrations in human plasma and its use in bioavailability studies is evaluated. Unchanged acyclovir has been quantified without the introduction of an internal standard using the present method. Human plasma proteins were selectively precipitated by the addition of 7% perchloric acid to spiked plasma samples or to the plasma samples obtained after acyclovir administration to human volunteers and the mixture was spun at 1000 *g* for 10 min. The supernatant was directly injected into a Novaflex C_{18} column and detected at 254 nm. The mobile phase consisted of octane sulfonic acid buffer (pH 2.5) and methanol (92:08). The limit of quantitation for acyclovir in plasma was 20 ng/ml, which enabled the determination of the area under the curve (AUC) more precisely, that is, it is much closer to its extrapolated value. The present method has been successfully applied to samples from bioavailability studies.
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1. Introduction

Acyclovir, 9-[(2-hydroxyethoxy)methyl]-9H-guanine, is an acyclic guanine nucleoside analog that lacks a 3′-hydroxyl on the side chain (Fig. 1). Acyclovir is a potent anti-viral agent useful in the treatment of Herpes Simplex Virus (HSV) infections. Acyclovir exerts its antiviral activity by competitive inhibition of viral DNA, through selective binding of acyclovir to HSV-thymidine kinase with about 200-fold greater affinity than for mammalian enzyme [1]. In vitro, it is most active against HSV-1 (0.02 to 0.9 μg/ml) and about two-fold less active against HSV-2 (0.03 to 2.2 μg/ml). The oral bioavailability of acyclovir ranges from 10 to 30% and the percentage decreases with increasing dose. Peak plasma concentrations average 0.4 to 0.8 μg/ml after 200 mg and 1.6 μg/ml after 800 mg dose [2]. The oral route is preferred to parenteral administration due to the risk of local toxicity at the injection site.

The elimination half-life ($t_{1/2}$) of acyclovir in adults with normal renal function ranges from 1.5 to 6.0 h, and is prolonged up to 20 h in anuric patients [3]. Renal excretion of unmetabolised acyclovir by glomerular filtration and tubular secretion is the

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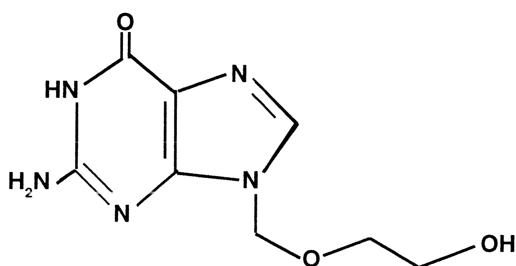

Fig. 1. Chemical structure of acyclovir: 2-amino-9[(2-hydroxymethoxy)methyl]1,9-dihydro-6H-purin-6-one.

principle route of elimination. Less than 15% is excreted as 9-carboxymethoxymethyl guanine or minor metabolites which are pharmacologically inactive. Previously reported methods for estimation of acyclovir in human plasma includes microbiological assay [4], ELISA technique [5], radioimmunoassay [6], scintillation proximity radioimmunoassay [7] and HPLC [8–14]. Previously reported HPLC methods involve time-consuming and costly extraction procedures with a minimum detectable concentration of about 225 ng/ml or use a fluorescence detection system at low pH conditions for achieving a detection limit of about 20 ng/ml.

This paper describes the development of a simple, economical, yet more sensitive HPLC method for estimation of acyclovir without the use of an internal standard. The method described was applied successfully to detect acyclovir in plasma samples obtained from a comparative bioavailability study carried out in healthy human volunteers after oral administration of a single 400-mg tablet. The major advantage of the present method is, using this method with a quantitation limit of about 20 ng/ml, it is possible to determine the pharmacokinetic parameter for the extent of absorption until the last sampling point ($AUC_{0\rightarrow t}$) more precisely so that it is much closer to its extrapolated value ($AUC_{0\rightarrow \alpha}$), as a difference of more than 20% between $AUC_{0\rightarrow t}$ and $AUC_{0\rightarrow \alpha}$ is not acceptable in the conclusion of bioequivalence.

2. Experimental

2.1. Materials

A reference standard of acyclovir was obtained from M/S Cheminar Drugs (Hyderabad, India). Two market brands of acyclovir tablets, Product A: Zovirax 400-mg tablet (Wellcome, South Africa) as the *reference* formulation and Product B: Vivorax 400-mg tablet (Cadila Pharmaceuticals, India) as the *test* formulation were used in the comparative bioavailability study. Perchloric acid was purchased from E. Merck (Mumbai, India). HPLC-grade methanol and octane sulfonic acid sodium salt were purchased from Spectrochem (Mumbai, India).

2.2. Equipment

The HPLC system LCM-1 (Waters) consisted of a series 600 pump, model 486 UV and 996 photodiode array detectors and an autosampler. The chromatographic data were analysed using Mellenium chromatographic manager version 2 (Waters). A C_{18} silica column (Novaflex, 300×4.6 mm I.D., 10 μm) from Flexit (Pune, India) was used for the HPLC separations.

2.3. Chromatographic conditions

The flow-rate was set to 1.5 ml/min and effluent was monitored with UV detection at 254 nm. The mobile phase composition was 8:92 (v/v) methanol and octane sulfonic acid buffer (0.05 *M* octane sulfonic acid sodium salt and pH adjusted with phosphoric acid to 2.5). All the analyses were performed at ambient temperature.

2.4. Sample preparation

Frozen plasma samples were thawed and brought to room temperature before they are processed for analysis.To a 1-ml aliquot of blank plasma or plasma spiked with known amounts of acyclovir, 1 ml of 7% (v/v) freshly prepared perchloric acid was added in a 6-ml centrifuge tube and thoroughly mixed by vortexing for 5 min. The precipitated plasma proteins were separated out by centrifuging this mixture for 10 min at 1000 *g* to get the drug in the supernatant. The supernatant layer was snapped and filtered through a Millex GV 0.22-μm filter unit into a HPLC sample vial directly. The vial was arranged in the autosampler and programmed to inject 100 μl of the sample into the chromatographic system. Unknown samples were prepared in an identical manner except for addition of acyclovir.

2.5. Method validation

The chromatographic method was validated on two different validation days to find out the accuracy precision and specificity of the present HPLC method for detection of acyclovir in plasma samples. The recovery of acyclovir from plasma was determined by comparing peak areas obtained from plasma to which acyclovir (20, 50, 100, 500, 1000 and 5000 ng/ml) had been added to that of the peak areas obtained from corresponding unspiked standards. The accuracy of the assay is defined as the absolute value of the ratio of back-calculated mean value of the validation samples to their nominal values and expressed as percentage. Precision is measured as the percent coefficient of variation over the concentration range of 0.02 to 5.0 μg/ml for acyclovir during the course of validation. Accuracy and precision on a single analytical day (intra-day) and on different days (inter-day) are shown in Table 1.

2.6. Bioavailability studies

Twelve healthy human volunteers with normal biochemical parameters were selected for the comparative bioavailability study after obtaining their

Table 1
Accuracy and precision with which acyclovir was detected on two separate validation days, during recovery studies and in quality control stability studies from spiked human plasma samples

Serial no.	Amount of acyclovir spiked (μg/ml)	Amount of acyclovir recovered (μg/ml)[a]		Accuracy		Precision	
		Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
1	5.000	Day 1: 5.000±0.026		100.0		0.52	
			5.011±0.140		100.23		2.80
		Day 2: 4.997±0.206		99.99		4.12	
		4.821±0.090[b]	–	96.37	–	2.04	–
		–	4.738±0.121[c]	–	94.75	–	2.55
2	1.000	Day 1: 0.999±0.013		99.98		1.31	
			0.998±0.012		99.98		1.23
		Day 2: 0.997±0.011		99.73	93.64	1.14	
		0.959±0.011[b]	–	95.92		1.04	–
		–	0.936±0.030[c]	–		–	3.23
3	0.500	Day 1: 0.462±0.082		92.42		0.48	
			0.4828±0.022		96.56		4.60
		Day 2: 0.503±0.007		100.68		1.51	
		0.473±0.006[b]	–	94.54	–	1.32	–
4	0.100	Day 1: 0.096±0.001		96.10	97.10	1.40	
			0.0971±0.002				1.91
		Day 2: 0.098±0.001		98.10		1.88	
		0.094±0.001[b]	–	94.16	–	1.11	–
5	0.050	Day 1: 0.054±0.001	0.0541±0.003	107.80	102.80	0.71	
							5.38
		Day 2: 0.049±0.001	–	97.60		2.90	
		0.048±0.002[b]		93.39	–	4.03	–
6	0.020	Day 1: 0.019±0.002	0.0197±0.002	96.58	–	8.14	
							7.98
		Day 2: 0.020±0.001	–	102.23		7.55	
		0.018±0.001		87.42	–	5.60	–
		–	0.019±0.001[c]	–	92.67	–	7.43

[a] Recovery values are back-calculated as % of acyclovir estimated in respective sample.
[b] Data from recovery studies in a single day ($n=6$).
[c] Data from quality control samples stored at −20°C during four week stability studies ($n=12$).

written, informed consent. Human subjects were aged 25±3.4 years (mean, ±SD) with a mean body weight of 54.85±6.2 kg (range 45 to 69 kg).The study protocol was discussed and approved by the Institutional Review Board and the Ethical committee of Cadila Pharmaceuticals, Research and Development unit. Two market brands of acyclovir 400 mg tablets (product A and product B) were selected for the study. One tablet each of either brand was administered to each volunteer (kept on overnight fasting) along with 200 ml of water such that, half the number of volunteers received product A and the remaining half received product B in the first part of a single blind, randomised two period *cross-over* study. The second part of the study was conducted after a wash-out period of 7 days, identical to part I, except that subjects who have received product A would now receive product B and vice-versa. Blood samples were obtained through in-dwelling venous catheter inserted into antecubital vein, prior to dosing (0 h and at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0 and 24.0 h after dosing. The blood samples were collected into heparinised glass tubes and immediately centrifuged at 500 *g* to separate plasma. The plasma samples were properly labelled and frozen at −20°C until analysis. The following pharmacokinetic parameters were determined to evaluate the two brands for their bioavailability and check for the bioequivalency. C_{max}, T_{max} are maximum plasma concentration and time to reach it respectively, obtained from the individual raw data. $AUC_{0\rightarrow t}$: area under the plasma concentration–time curve during the sampling period, determined by linear trapezoidal rule. K_{el}: elimination rate constant, calculated as the slope of the log-linear regression line in the terminal part of elimination phase.

$AUC_{0\rightarrow\infty}$: extrapolated area under the curve calculated as $AUC_{0\rightarrow t}+C_{last}/K_{el}$, where, C_{last} is the last measurable concentration. MRT: mean residence time calculated as $AUMC_{0\rightarrow\infty}/AUC_{0\rightarrow\infty}$, where $AUMC_{0\rightarrow\infty}$ is the area under the first moment curve from zero to infinity. $T_{1/2}$: elimination half-life calculated as $0.693/K_{el}$. The pharmacokinetic parameters viz., $C_{max,}$ $T_{1/2,}$ $AUC_{0\rightarrow t}$ $AUC_{0\rightarrow\infty}$ and MRT obtained after product A and product B were compared using Anova and two-sided paired Student's *t*-test. Wilcoxon signed rank test was used to compare T_{max} values.

The 95% confidence intervals were determined for the pharmacokinetic parameters resulting from product A in comparision with those from product B. Statistical significance was defined at the level of $P\leq0.05$.

3. Results and discussion

3.1. Sample preparation

Since acyclovir is freely soluble in aqueous environment, the plasma proteins and other nucleic acids were precipitated at pH<2.5. Some organic solvents such as dichloromethane, chloroform and *n*-hexane were tried for extraction. The recoveries were found to be much less and very high processing losses were encountered compared to using the above-described method which involves a single step and simple procedure, resulting in good recoveries.

3.2. Selectivity

No peaks interfered with the detection of acyclovir in spiked plasma samples with that of the plasma components as can be seen in representative chromatograms in Fig. 2. The peak purity of corresponding acyclovir peaks in spiked plasma samples was confirmed by analysing the peak spectra using a photodiode array detector (PDA) and Mellenium software. This further confirms the selectivity of the method.

3.3. Linearity

The small percentage of differences between nominal and found concentrations of the standards in the standard curves (Table 1) for both intra-day and inter-day data suggest that the assay method is linear over the entire concentration range investigated. The intra-day regression was found to be more than 0.9998.

3.4. Precision and accuracy

Both intra-day and inter-day accuracy and precision of the standard curve were examined. As can be seen from Table 1 and the fact that C.V. values were

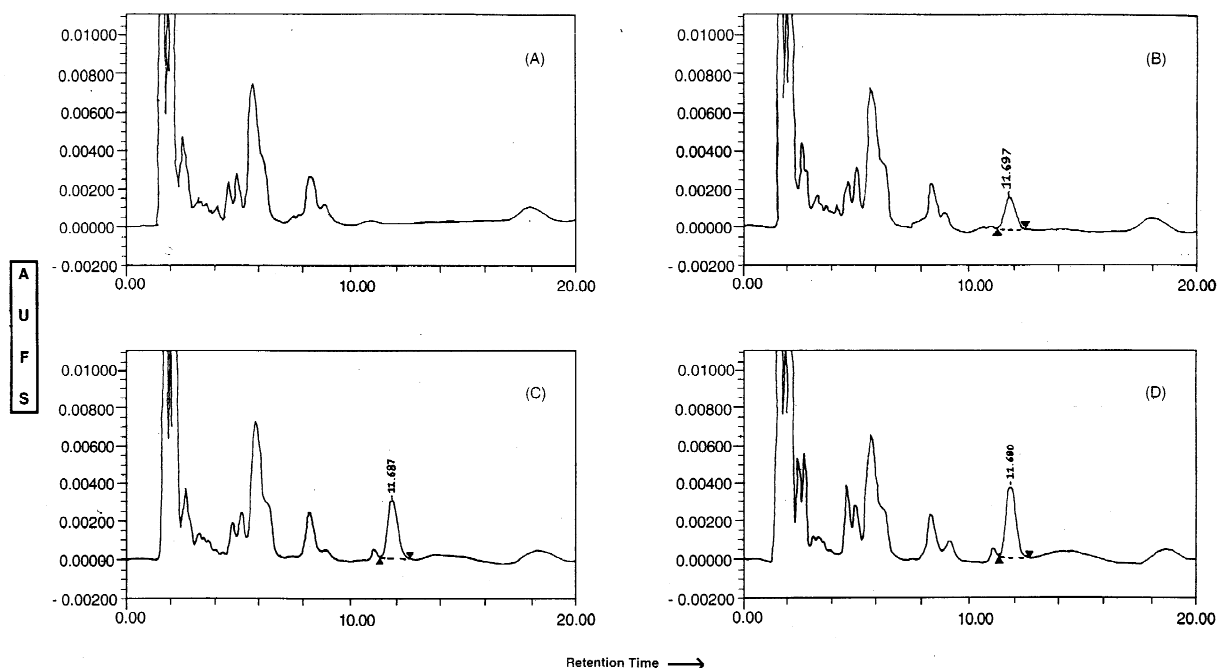

Fig. 2. Representative chromatograms of blank plasma, plasma spiked with acyclovir and acyclovir in plasma samples obtained from human subjects after oral administration of acyclovir tablets. (A) Control blank plasma, (B) control plasma spiked with acyclovir 0.50 μg/ml, (C) plasma levels at 1.5 h post dosing in subject 5, (D) plasma levels at 1.5 h post dosing in subject 6.

all less than 10%. Furthermore the small percentage of difference between nominal and found concentrations of the standards showed that the assay was sufficiently accurate for its application in biostudies.

3.5. Stability

Analysis of plasma stability control samples studied at concentrations of 20 and 5000 ng/ml over a period of four weeks. The results (Table 1) showed that acyclovir was stable when stored at −20°C. Mean concentrations detected were between 92 to 95% of nominal. Stability studies beyond four weeks were not carried out as there was no relevance.

3.6. Recovery

The mean absolute recovery of the analyte from plasma ranges from 87.42 to 96.37% for acyclovir. The recovery was calculated by comparing peak areas from un-extracted standards with those of extracted standards, across the range of each standard curve.

3.7. Limit of quantitation

The limit of quantitation (intra-day C.V.≤10%) for acyclovir in plasma was found to be 20 ng/ml. This concentration limit covers more than 98% of the plasma concentrations–time curve in the present bioavailability study.

3.8. Analysis of clinical samples from bioavailability study

The method described has been successfully applied for the quantitation of acyclovir in about 260 plasma samples resulting from the comparative bioavailability study of acyclovir-400 tablets. The mean concentration profile obtained for twelve human volunteers following the oral administration of product A and product B is shown in Fig. 3. The maximum concentration (C_{max}), the area under the

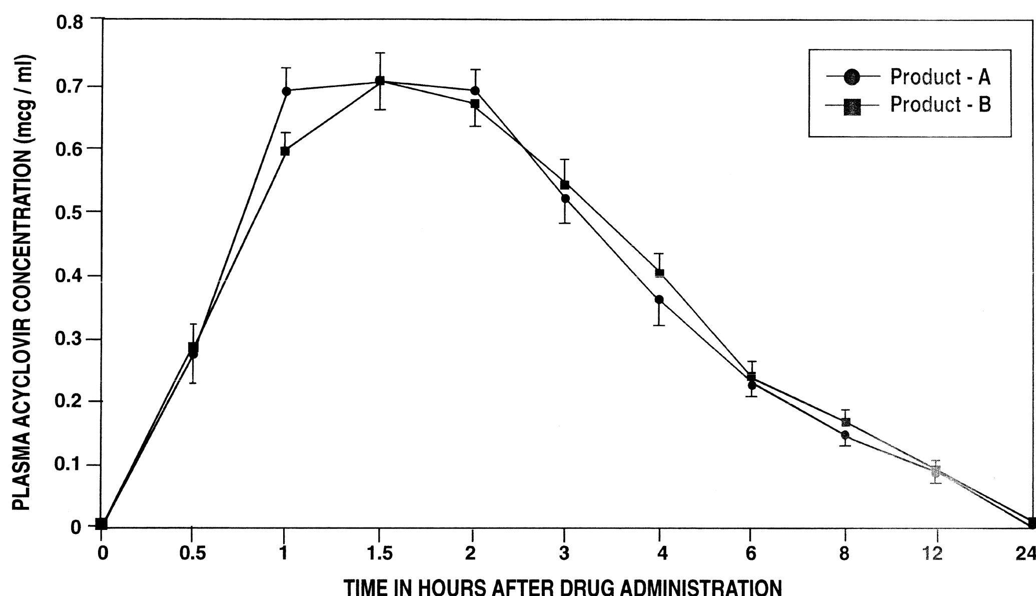

Fig. 3. Mean plasma acyclovir concentration–time profile following single oral administration of 400 mg acyclovir of either *reference* or *test* brand in twelve healthy human volunteers.

curve (AUC) and mean residence time (MRT) for the two preparations were comparable and were not significantly different (Table 2). The time for maximum concentration (T_{max}) for both the preparations ranged from 1.0 to 3.0 h. The pharmacokinetic parameters were subjected to two-way Anova and 90% confidence intervals were determined for product A in comparison with product B using different

Table 2
Mean pharmacokinetic parameters and the relevant statistical analysis

Serial no.	Pharmacokinetic parameter studied	Pharmacokinetic parameter mean±SD obtained with		Ratio B/A	P-value[a] Anova/ paired t-test	95% Confidence intervals based on			
		Product A	Product B			Anova (normal)	Anova (log data)	Westlake (normal)	Westlake (log data)
1	C_{max} (μg/ml)	0.830 ±0.235	0.817 ±0.237	1.019 ±0.251	NS	83.22–113.59	83.93–116.43	84.31–115.69	83.13–117.55
2	AUC $_{0\rightarrow 24}$ (μg h ml $^{-1}$)	3.394 ±1.120	3.512 ±1.243	1.064 ±0.229	NS	90.09–117.48	89.79–107.82	92.18–107.82	91.78–108.42
3	AUC $_{0\rightarrow \infty}$ (μg h ml $^{-1}$)	3.631 ±1.167	3.788 ±1.378	1.055 ±0.219	NS	90.18–117.23	89.79–118.96	92.24–107.26	91.78–108.42
4	$T_{1/2}$ (h)	3.772 ±0.934	3.544 ±0.603	1.066 ±0.212	NS	80.45–116.40	80.08–117.40	81.64–118.36	80.85–119.42
5	CL (l/h)	106.920 ±30.29	116.837 ±37.33	0.936 ±0.156	NS	81.20–115.70	80.89–116.20	81.90–118.10	80.36–119.08
6	M.R.T (h)	5.571 ±0.903	5.228 ±0.960	1.078 ±0.140	NS	83.95–114.15	83.38–114.40	85.23–114.57	84.75–115.40
7	T_{max}[b] (h)	1.417 ±0.571	1.542 ±0.594	1.153 ±0.399	NS				

[a] NS=No significant difference (P<0.05).
[b] Test of significance was determined by Wilcoxon signed rank test and found no significant difference at P=0.05.

statistical methods as in Table 2. The results indicate that the pharmacokinetic profiles resulting from the two-products were identical leading to conclusion of bioequivalency of the products tested. Prior to initiation of biostudy the marketed products were analysed for acyclovir label content using a HPLC method routinely used in our laboratory for the analysis of acyclovir tablets. The contents of acyclovir were found to be 99.25±1.25 and 98.75±1.32 for products A and B respectively.

4. Conclusions

The proposed HPLC method has been shown to measure precisely the acyclovir concentrations following single oral administration of acyclovir 400-mg tablet in human volunteers. The method has been found to be linear and reproducible over the entire range of clinically significant and therapeutically achievable plasma concentrations. The pharmacokinetic parameters obtained using the present analytical method were found to be very much in agreement with the corresponding values cited in the literature [3]. In most cases the plasma concentrations fell below the detection limit at 24 h and the therapeutic concentrations were maintained up to 8 h following a single oral dose of a 400-mg tablet. The AUC ratios in the sampling period to their corresponding extrapolated values were found to be between 91 to 96%, indicating the usefulness of the sensitivity achieved by the present method in quantification of unchanged acyclovir in plasma samples from bioavailability studies.

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