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# Short Communication

# New, high-sensitivity high-performance liquid chromatographic method for the determination of acyclovir in human plasma, using fluorometric detection

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ABSTRACT

A high-performance liquid chromatographic method for the determination of acyclovir in human plasma has been developed. It is the first published chromatographic method capable of determining acyclovir in plasma with sufficient sensitivity and for sufficiently long periods of time following oral administration of a standard dose of acyclovir during pharmacokinetic investigations. Following precipitation of the proteins with perchloric acid, the sample is chromatographed with a strongly acidic mobile phase on a reversed-phase column, and is then subjected to fluorometric detection (excitation 260 nm, emission 375 nm). The determination limit is 6–10 ng/ml human plasma. The calibration is linear in the range 10–12 400 ng/ml plasma, with the coefficients of variation less than 8%. The absolute recovery rate is between 102 and 113%. This method has already been used to analyse several thousand plasma samples.

## INTRODUCTION

Acyclovir, 9-(2-hydroxyethoxy)methylguanine (Fig. 1), is a synthetic purine nucleoside analogue derived from guanine. This drug differs structurally from guanine by reason of its N-glycosidically conjugated acyclic side-chain, which is structurally similar to the cyclic, natural nucleoside 2′-deoxyguanosine. Owing to the absence of carbon atoms from the 2′ and 3′ positions, the acyclic guanosine analogue cannot participate in DNA synthesis.

Acyclovir has an anti-viral action. Of all such known substances, acyclovir stands out as unique in that it inhibits virus replication in the infected

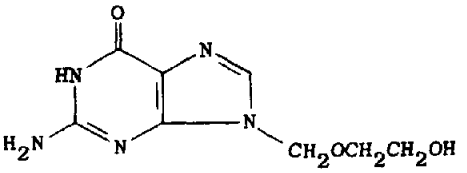


Fig. 1. Structure of acyclovir.

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cell, in a highly potent and highly selective manner, without affecting the functions of non-infected cells. After being absorbed into the infected cell, acyclovir is first metabolized into acycloguanosine monophosphate by means of a viral thymidine kinase. In further steps, the cell's own kinases then phosphorylate the monophosphate into a triphosphate, this being the actual active substance. The triphosphate inhibits the virus-specific DNA polymerase (thus also inhibiting virus replication), while the body's own cellular DNA polymerase is inhibited by a factor of only between 10 and 30 times less. The anti-viral action of acyclovir is thus confined to viruses whose replication is dependent on viral-coded enzymes, mainly thymidine kinase.

Acyclovir is thus only effective against viruses of the herpes group, being particularly effective against *Herpes simplex* virus Type 1 and Type 2 infections. In high concentrations, achieved by intravenous infusions, it is also effective against varicellazoster virus infections.

Sensitive determination is necessary in order to obtain more exact information on the pharmacokinetics of this substance. Acyclovir is administered to humans both intravenously and, more commonly, orally. Following oral administration of a standard dosage, maximum concentrations of 600–1200 ng/ml are found in human plasma [1,2]. As the elimination half-life is *ca.* 3 h, it is frequently the case, both in clinical studies on patients and in pharmacokinetic investigations with test subjects, that concentrations of less than 100 ng/ml of plasma are measured in many plasma samples. Moreover, international recommendations stipulate a determination limit of less than 5% of $C_{max}$ for pharmacokinetic investigations [small difference between area under the curve (AUC) and AUC $_{0-inf}$]. Previous publications on the determination of acyclovir have used radioimmunoassay (RIA) [3], enzyme-linked immunoassay (ELISA) [4] and high-performance liquid chromatography (HPLC) [5–9], with detection being performed using UV [5,6,8,9] or fluorescence [7]. Only the RIA method [3], however, has a sufficiently sensitive determination limit (11 ng/ml of plasma). All the other methods have determination limits of 200–1200 ng/ml plasma, which is nowhere near sufficient for pharmacokinetic investigations in which 200–800 mg of acyclovir are orally administered.

The method described here has a very low limit of determination, following precipitation of the proteins and reversed-phase chromatography with fluorometric detection.

## EXPERIMENTAL

### *Apparatus and chemicals*

The HPLC equipment comprised an HP-1090 M liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with a Merck-Hitachi F 1000 fluorometric detector (time constant, 1 s; bandwidth, 15 nm) (Merck, Darmstadt, Germany) and a PE-Nelson 2600 data system (Perkin Elmer, Cupertino, CA, USA). The solvents and reagents used were of HPLC grade from Rathburn (Walkerburn, UK) or of analytical grade from Merck. The analytical column was filled with Nucleosil 120 $3C_{18}$ (80 mm × 4 mm I.D., particle size 3 μm, SRD-Pannosch, Vienna, Austria).

### *High-performance liquid chromatography*

The mobile phase consisted of two different components, with 0.02 *M* perchloric acid being pumped during the period 0–3 min, followed by 0.02 *M* perchloric acid–acetonitrile (55:45, v/v) for 3 min, in order to clean the column. This cleaning step was done after each sample injection. The flow-rate was 1.5 ml/min and the column temperature was maintained at 30°C. Fluorometric detection took place at an excitation wavelength of 260 nm and an emission wavelength of 375 nm. The injection volume was 20 μl. The retention time for acyclovir was *ca.* 2.3 min.

### *Sample preparation*

After the plasma samples (EDTA, heparin, citrate) had been thawed, 1 ml of sample was added to 0.3 ml of 3 *M* perchloric acid and mixed for 15 s on a vortex-mixer. Following centrifugation (2 min, 2000 *g*), 0.5 ml of the clear supernatant was transferred to an autosampler vial; of this, 20 μl were analysed.

*Validation*

The method was validated by adding various amounts of acyclovir to pooled human plasma. The resulting concentrations were 10.3, 24.8, 82.6, 206.4, 495.4, 1032.0, 2580.0, 6192.0 and 12 384 ng/ml of plasma. These calibration samples were used to test the linearity and precision of the method. At concentrations of 24.8, 206.4, 1032 and 6192 ng/ml, the absolute recovery rate was tested by comparison with directly injected aqueous standard solutions.

For testing the specificity, plasma samples from ten different volunteers were analysed during the method development.

## RESULTS AND DISCUSSION

*Chromatography*

From the previous papers on HPLC and UV detection, as well as from a number of preliminary experiments, it was apparent that UV detection was not suitable for achieving the desired determination limit of 10–20 ng/ml of plasma. Owing to the pronounced hydrophilicity of acyclovir, it is impossible to extract it from plasma using organic solvents with a sufficient degree of recovery, and thus to pre-purify and concentrate it. This property of acyclovir also rules out liquid–solid prepurification on small reversed-phase clean-up columns (off-line or on-line). It was thus first necessary to improve the detectability in order to make it possible for absolute amounts to be determined in the picogram range. The work performed by Salamoun *et al.* [7] provided a good starting point, although it became apparent that the fluorescence was very heavily pH-dependent. Under the separation and detection conditions employed by Salamoun *et al.*, an absolute detection limit of 500 pg was found. We were able to reduce this value to *ca.* 50 pg.

Working on the basis that the mobile phase would have to be very acidic (fluorescence optimization), we were able, after several preliminary experiments, to find a separation column and a mobile phase capable of eluting acyclovir ahead of other (endogenous) purine derivatives and numerous other unwanted substances.

After acyclovir has been eluted, the unwanted substances that elute later are washed from the column by step-gradient elution. Under the HPLC conditions used, a sample may be injected every 10 min.

*Validation*

*Linearity and determination limit.* After analysis of the first validation samples, the linear regression between spiked plasma concentrations and the respective peak areas was determined. With multiple analysis ($n = 26$) in the 10.3–12 384 ng/ml calibration range, there was a slope of 323.588 with an intercept of 425.9 and a correlation coefficient ($r^2$) of 0.9999 (regression weight $1/x$). Calibrations performed on several different days resulted in a slope of 212.501 ± 9.7% ($n = 14$). The slope changes are a result of changes in the injection volume.

During routine analysis a typical value computed for the detection limit was 5.5 ng/ml of plasma [10] and for the determination limit was 8.2 ng/ml. The coefficient of variation (C.V.) at the calibration point 8.9 ng/ml of plasma was ±5.3%, and the accuracy was +8.1%.

*Precision and accuracy.* The C.V. were between 4 and 8% in the 10–80 ng/ml range, and below 1% in the 200–12 000 ng/ml range. The accuracy was also excellent between +4.0% and −0.4%, despite the fact that the calibration was performed over a range of more than 1:1000.

*Recovery.* The recovery rate, determined at four different concentrations, was between 102.1 and 112.9% (Table I).

*Reproducibility.* The analysis of quality control plasma samples spiked with acyclovir on four days resulted in a reproducibility ($n = 3$) of 12.68% at 20.8 ng/ml, 1.65% at 201.2 ng/ml and 3.88% at 2073.6 ng/ml (Table II).

*Specificity.* Plasma samples from ten volunteers who had not taken acyclovir administration showed no interfering peaks around the retention time of acyclovir.

*Stability of acyclovir in plasma*

After a storage period of three months at −20°C, plasma samples containing acyclovir

TABLE I

RECOVERY OF ACYCLOVIR FROM HUMAN POOL PLASMA

| Concentration in aqueous solution, direct injection (ng/ml) | $n$ | Concentration found in spiked plasma (ng/ml) | C.V. (%) | Recovery (%) |
|---|---|---|---|---|
| 24.8 | 5 | 28.0 | 4.94 | 112.9 |
| 206.4 | 5 | 216.0 | 1.19 | 104.7 |
| 1032.0 | 5 | 1057.0 | 2.17 | 102.4 |
| 6192.0 | 5 | 6324.0 | 2.41 | 102.1 |

from a clinical study were analysed again. The results showed a deviation from −1.0% to +17.2%, with a median of +4.22% (concentration range: 54.7–248.6 ng acyclovir per ml plasma).

*Plasma samples*

In the course of pharmacokinetic investigations, thousands of samples have already been analysed using this method. Fig. 2 shows a selection of these chromatograms.

TABLE II

DAY-TO-DAY VARIATION OF QUALITY CONTROL PLASMA SAMPLES

| Day | Plasma sample 1 | | Plasma sample 2 | | Plasma sample 3 | |
|---|---|---|---|---|---|---|
| | Concentration observed (ng/ml) | Accuracy (%) | Concentration observed (ng/ml) | Accuracy (%) | Concentration observed (ng/ml) | Accuracy (%) |
| 1 | 19.4 | −5.79 | 199.8 | −2.77 | 1963.7 | −4.45 |
| | 20.7 | 0.83 | 198.6 | −3.34 | 1960.9 | −4.58 |
| | 20.8 | 0.97 | 198.4 | −3.46 | 1968.6 | −4.21 |
| 2 | 18.6 | −9.73 | 201.9 | −1.74 | 2054.5 | −0.02 |
| | 17.9 | −12.90 | 200.7 | −2.36 | 2064.0 | 0.44 |
| | 23.8 | 15.77 | 197.3 | −3.97 | 2023.6 | −1.53 |
| 3 | 22.0 | 6.96 | 201.5 | −1.95 | 2173.4 | 5.76 |
| | 22.0 | 6.86 | 207.5 | 0.98 | 2133.8 | 3.84 |
| | 25.9 | 26.23 | 205.3 | −0.12 | 2108.3 | 2.59 |
| 4 | 17.5 | −14.99 | 205.1 | −0.20 | 2142.5 | 4.26 |
| | 18.4 | −10.51 | 201.7 | −1.83 | 2181.8 | 6.17 |
| | 23.3 | 13.38 | 197.1 | −4.11 | 2108.6 | 2.61 |
| $n$ | 12 | 12 | 12 | 12 | 12 | 12 |
| Mean | 20.8 | 1.42 | 201.2 | −2.07 | 2073.6 | 0.91 |
| C.V. (%) | 12.68 | | 1.65 | | 3.88 | |

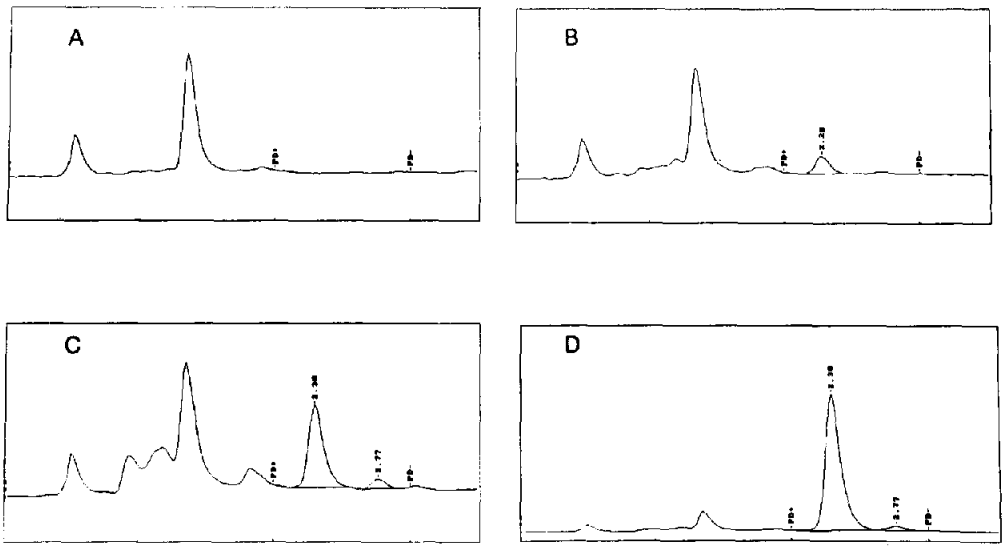


Fig. 2. Chromatograms of plasma samples taken after oral administration of 400 mg of acyclovir. The retention time of acyclovir is *ca.* 2.3 min. (A) Subject 2, before administration. (B) Subject 2, 15 h after administration: 23.2 ng/ml. (C) Subject 3, 10 h after administration: 112.7 ng/ml. (D) Subject 2, 3 h after administration: 1181.9 ng/ml.

## CONCLUSION

The analytical method described here is the first published HPLC method capable of determining concentrations of acyclovir in human plasma for a sufficiently long period of time following oral administration of a standard dose. The limit of determination is *ca.* 6–10 ng/ml; in connection with dermal systematic availability tests with acyclovir, this limit has recently been reduced still further to 2 ng/ml, by increasing the injection volume (Fig. 3). Fig. 4 shows the plasma concentration of a representative test subject af-

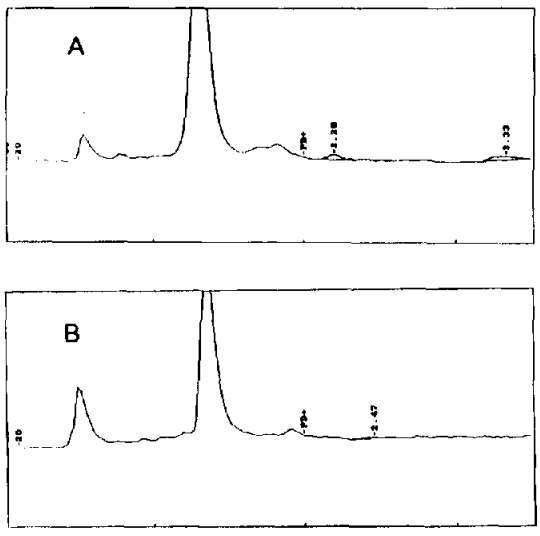


Fig. 3. Chromatograms of plasma samples taken after dermal application of acyclovir cream. The retention time of acyclovir is *ca.* 2.3 min. (A) Subject 5, after dermal application: 2.04 ng/ml. (B) Subject 5, before dermal application.

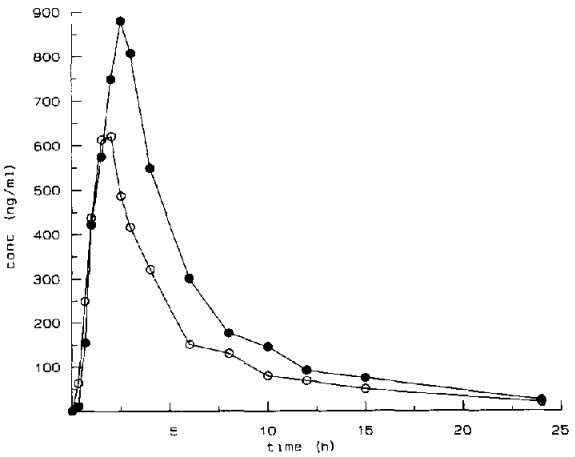


Fig. 4. Plasma concentrations of a representative volunteer after oral administration of 200 mg of acyclovir in two different galenic formulations.

ter oral administration of 200 mg of acyclovir in two different galenic formulations.

The key features of this method are the selection of the mobile phase and stationary phase (elimination of endogenous purines) and the fact that fluorometric detection is performed under highly acidic conditions (*i.e.* increase in sensitivity).

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