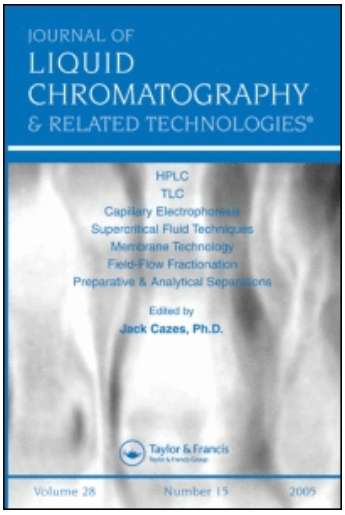

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RAPID DETERMINATION OF ACYCLOVIR IN PLASMA BY REVERSED PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high performance liquid chromatographic method has been developed for the determination of a new antiviral drug : Acyclovir [9-(2 hydroxyethoxymethyl) guanine] , in human plasma, using a reversed-phase method, with ultraviolet detection at 254 nm.

The internal standard, acetaminophen, was used as an aid to quantitation. The plasma sample was deproteinized with acetonitrile and the clear obtained supernatant was cleanup with methylene chloride, then directly analyzed in the chromatographic system. The limit of detection was about 0.25 µg/ml of plasma with a precision of $\pm$ 7.4 %.

The method is used for quantitative acyclovir in plasma from renal failure patients, with satisfactory accuracy and precision. Endogenous substances and a variety of drugs concomitanly used in antiviral therapy did not interfere with the assay.

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INTRODUCTION

Acyclovir (Figure 1), a new antiviral agent with a high level of activity against herpes virus (1, 2) is used for a few years in the United States and in Europe. Several methods have been described in pharmacokinetic studies (3, 4, 5) and for the monitoring of plasma concentrations of acyclovir in patients with renal failure (6, 7, 8). Radioimmunoassay methods (9, 10, 11, 12) depend on the purity and specificity of the antibodies. Interferences and cross reactions have been reported for both techniques (9, 10, 11).

High-performance liquid chromatography (H.P.L.C.) has been used (13, 14, 15). The ion-exchange procedure (13), is limited by a poor efficiency and a low number of analyses performed in a working day.

The liquid chromatographic methods using reversed-phase (14, 15) were very specific and have a good recovery, but the chromatographic peak of acyclovir was poorly resolved from the endogenous peaks at low concentration. The high performance liquid chromatographic method described here, was designed to provide an accurate, sensitive, specific and rapid acyclovir assay, which can be performed for use in therapeutic monitoring and for pharmacokinetic and disposition studies.

MATERIAL AND METHODS

Reagents and materials :

Acyclovir and acetaminophen (i.s), were supplied by Wellcome laboratories - Monaco - France and Bottu laboratories - Nanterre - France, respectively.

Water was deionized then doubly distilled and filtered through a 0.22 um - type GC - cellulose ester filter, Millipore corp., Bedford, Mass. 01730.

Standard stock solutions of acyclovir (100 ug/ml and acetaminophen (200 ug/ml), were prepared in water and stored subsequently at + 4°C for a week, in the dark. The appropriate concentrations of standard solutions were prepared by diluting the stock solutions in water. H.P.L.C. grade acetonitrile, potassium dihydrogen phosphate, methylene chloride were purchased from Carlo-Erba - Milan - Italy and H.P.L.C. grade methanol, so-

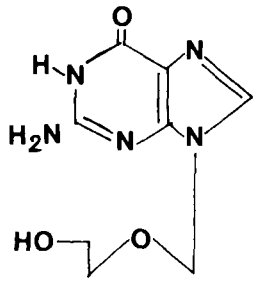


FIGURE 1 : Chemical structure of Acyclovir.

dium hydroxyde 30 % R.P., sodium chloride GR, phosphoric acid 85 % R.P., chlorhydric acid 38 %, heptan sulfonic acid, were purchased from Merck - Darmstadt - G.D.R.

Apparatus :

H.P.L.C. system consisted of a model 6000 A solvent delivery system and a model 710 A wisp sample injector (Waters Associates, Inc., Milford - Mass - 01757 - U.S.A.). A model 440 absorbance detector operating at 254 nm (Waters Associates), with the absorbance detector sensitivity set at 0.005 absorbance units full scale (a.u.f.s.), for plasma samples.

The chromatograms were recorded on an Omniscribe (Houston Instrument - Gistel - Belgium). The mobile phase was prepared daily. It consisted of 0.25 ml acetonitrile in 999.75 ml 0.01 M potassium dihydrogen phosphate buffer (pH 2.3) containing 0.9 g heptan sulfonic acid. The solution was filtered through 0.22 µm filter. Under these conditions, acyclovir and the internal standard were eluted with retention times of 9.3 min. and 7.9 min. respectively, as illustrated in figure 2 and carried through an octadecylsilan µ-Bondapack C_{18} column (30 cm x 3.9 mm, particle size 10 µm, Waters Assoc.) at 1.0 ml/min and ambient temperature.

Extraction procedure :

In a 10 ml screw-capped tube, 1.0 ml of plasma (sample to be assayed or standard), was supplemented with 100 µl of an aqueous internal-standard solution (200 µg/ml) and 100 µl 0.1 M hydrochloric acid saturated with sodium chloride, then homogeni-

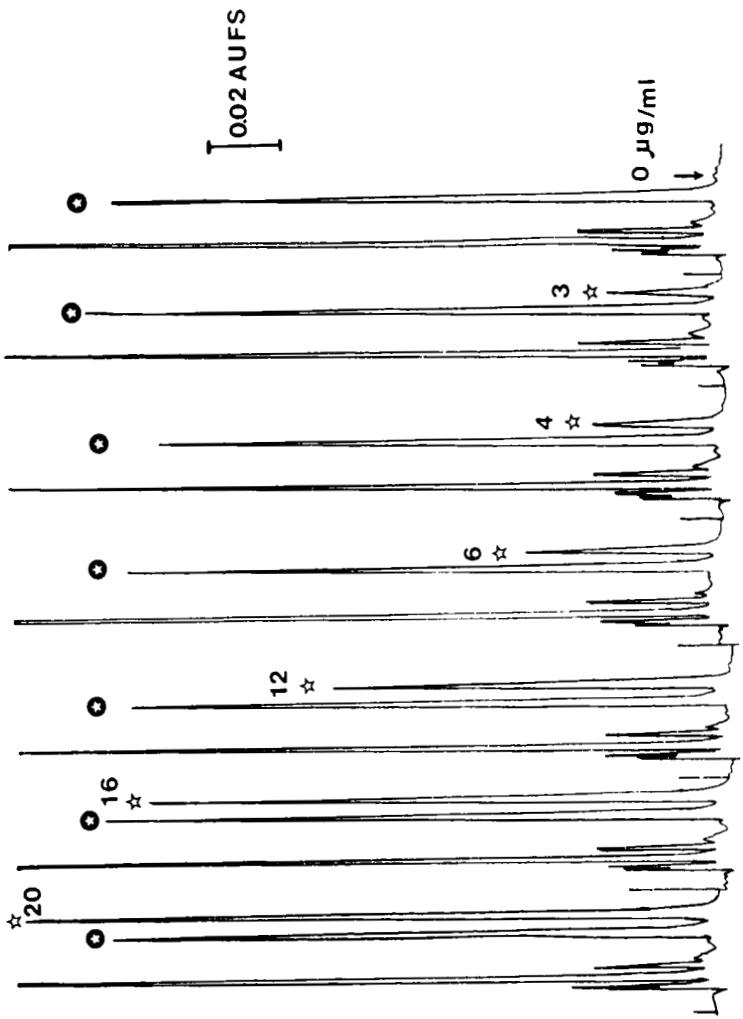


FIGURE 2 : Chromatogram of Acyclovir (☆) and internal standard (✪).
(loop : 20 µl, S : 0.02 a.u.f.s.)

zed by slow rotation. After addition of 2.0 ml acetonitrile, the tube was vortex-mixed vigorously for 1 min., then centrifuged at 2000 g. for 10 min..

An 2.5 ml aliquot of the supernatant was transferred to another glass tube and mixed with 6.0 ml of methylene chloride. The two phases were separated by centrifugation for 10 min. at 2000 g. An aliquot (30 µl) of aqueous layer was injected into the H.P.L.C. system.

Calibration :

Two standard concentration curves (Figure 3) were obtained by adding : 0 - 0.25 - 0.50 - 1.0 - 2.0 - 3.0 - 4.0 µg/ml of acyclovir, then 50 µl of acetaminophen (i.s) (200 µg/ml) and 3.0 - 4.0 - 6.0 - 12 - 16 - 20 µg/ml of acyclovir then 100 µl of (i.s 200 µg/ml) (Figure 2), in control plasma under the same experimental conditions.

RESULT AND DISCUSSION

We found a linear correlation between the concentration of acyclovir and the ratio of peak heights : acyclovir/i.s., in ranges of the two standard curves (Figure 3).

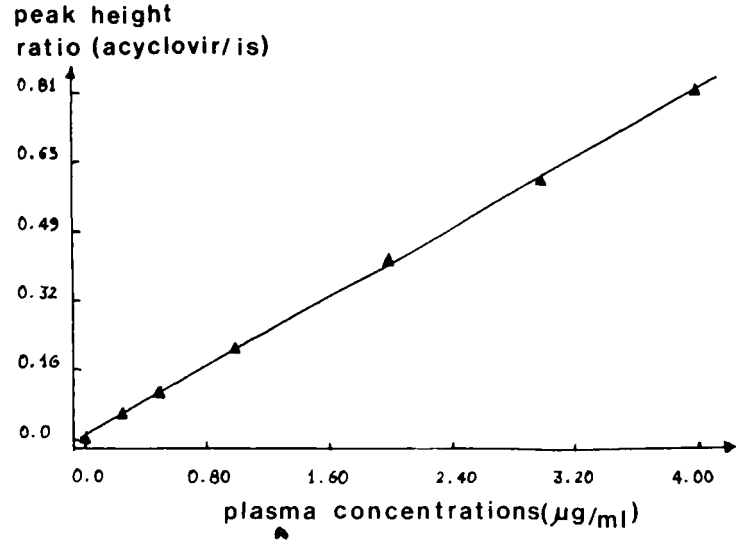
The equations describing the standard curves, determined by linear least-squares regression analysis were : $y = 0.057 X - 0.001$ and $y = 0.20 x - 0.008$ for ranges [3.0 - 2.0] and [0.25 - 4.0] µg/ml, respectively. The corresponding correlation coefficients (r) were 0.9997 and 0.9998.

The intra-assay precision of acyclovir was assessed by repeated analysis of fresh drug-free human specimens spicked with known concentrations of acyclovir.

As shown in table I, the within-day precision of the method was illustrated by the coefficient of variation : 3.39 % and 7.38 %, for 6.0 and 0.25 µg/ml respectively.

As can be seen from Figure 4, no endogenous peak extracted from the blank plasma interferes with that of acyclovir and i.s., which is well below the detection limit.

The limit of detection of this method was 0.25 µg/ml, allowing a signal-noise ratio of 3, when 1.0 ml of plasma was used (Figure 5). This value was the same, that obtained previously (14, 15), but the eluent and the extraction method used here



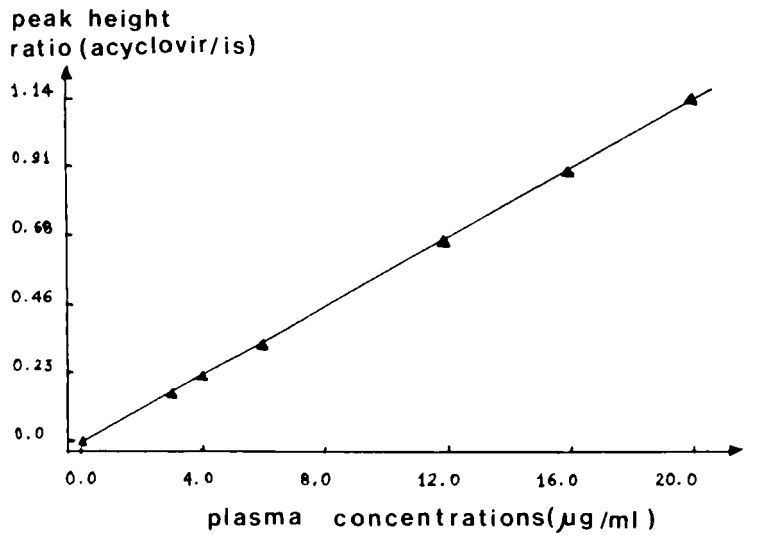


FIGURE 3 : Linear regression analysis of Acyclovir, in ranges of two standard curves.

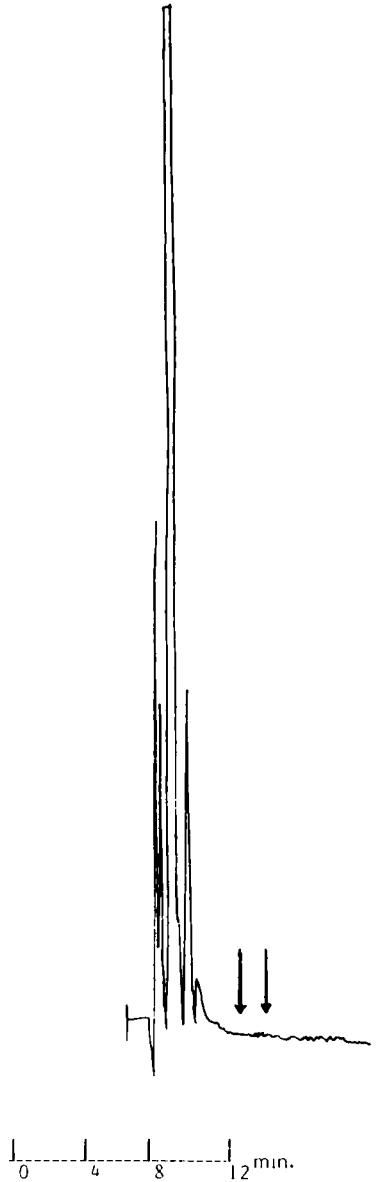


FIGURE 4 : Chromatograph of human drug free plasma extract.
(loop : 20 µl, S - 0.01 a.u.f.s.)

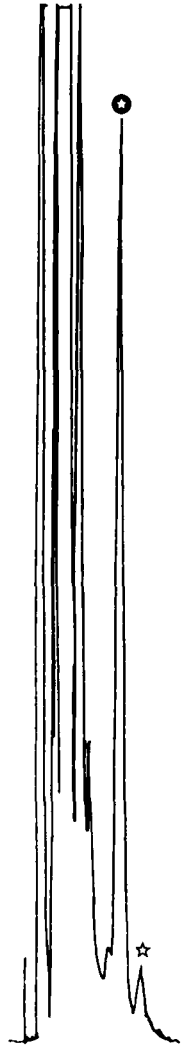


FIGURE 5 : Chromatogram from a spiked plasma sample (0. 25 µg Acyclovir/ml) showing the detection limit of the method.
(loop : 20 µl, S = 0.01 a.u.f.s.)
(☆) Acyclovir (✪) i.s.

TABLE I : INTRA-ASSAY PRECISION ACYCLOVIR IN HUMAN PLASMA

Spiked concentration (µg/ml)	Measured concentration Mean ± s.d (µg/ml)	CV (%)
0.25 (n = 12)	0.245 ± 0.018	7.38
6.0 (n = 10)	6.09 ± 0.206	3.39

TABLE II : INTER ASSAY PRECISION (REPRODUCTIBILITY) IN PLASMA (n = 6)

Amount added (µg/ml)	Amount found mean (± s.d)	CV (%)	Recovery (%)
2.0	1.98 ± 0.10	5.1	99.0
4.0	4.05 ± 0.20	4.9	101.3
6.0	5.99 ± 0.22	3.65	99.8
8.0	8.01 ± 0.22	2.8	100.1
16	16.00 ± 0.51	3.2	100.0
20	20.03 ± 0.68	3.4	100.2

TABLE III : STABILITY OF ACYCLOVIR IN FROZEN PLASMA SAMPLES

Week	Amount added 2 µg/ml	Concentration found (µg/ml)	Amount added 10 µg/ml	Concentration found (µg/ml)
1	"	1.6	"	10.0
2	"	1.9	"	10.1
3	"	2.0	"	11.2
		2.0	"	12.5
4	"	2.1	"	10.6
Mean ± S.D.		1.93 ± 0.18		10.97 ± 0.94
CV %		9.06		8.57

were, however, found to lead to a minimum of interference in the analysis. Using phosphate buffer and counter-ion, no endogenous peak was detected.

The data presented in table II, shows the precision and accuracy of this assay. A good reproductibility was determined at the same concentrations (6), in six replicate runs, over a period of one month.

No interfering peak were observed in the plasma of patients receiving acyclovir in combination with drugs such as : amikacin, cefazolin, 5-flucytosine, ticarcillin, amphotericin B, aracytine, vidarabine, cis-plastinum, vincristine, methotrexate, 5-fluoro-uracil, melphalan, caffeine.

The life time of the column appears to be very good, as it is still in excellent condition after four months use. The sensitivity of RIA assay (9, 10, 11, 12) is more important (0.01

µg/ml), than our H.P.L.C method (0.25 µg/ml), but cross-reactions with thioguanin compounds, and pre-dilution steps are necessary in RIA assay (3, 4).

No decrease in the measured acyclovir concentration was detected, when samples were stored for periods of up to four weeks at - 20°C (Table III).

As compared to the other H.P.L.C. methods, is its specificity and rapidity.

In conclusion, the proposed H.P.L.C. procedure for the quantitative determination of acyclovir in human plasma offers appreciable accuracy, precision and rapidity. The method is easily applicable to the monitoring of plasma concentrations of drug in patients with renal failure and is also sufficiently sensitive for its potential use in pharmacokinetics and bioavailability studies, after a single administration.

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