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Technique validation by liquid chromatography for the determination of acyclovir in plasma

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Abstract

In this research project, a high-performance liquid chromatography (HPLC) method was developed for the determination of acyclovir (ACV) in plasma. The plasma samples, recharged with acyclovir and in presence of 5′-*N*-methylcarboxy-amidoadenosine (MECA) as an internal standard, were purified using a solid-phase extraction technique with Waters Oasis HLB columns. The separation of the components from the extract was carried out in a LiChrospher 100 RP-18 column for further ultraviolet detection at a wavelength range of 250–260 nm. The mobile phase composition was 18% acetonitrile, sodium dodecylsulphate 5 m*M* and phosphate buffer at pH 2.6 with an analysis time of 13 min per sample. The average retention time for acyclovir was of 5.0 min and for the internal standard 11.2 min. The calibration curve was linear ranging between 0.05 and 1.80 μg/ml. The detection limit was 0.006 μg/ml with a quantification limit of 0.020 μg/ml. The ACV recuperation percentage for 250 μl of plasma was between 94.7 and 109.7% with a coefficient of variation not higher than 5.2%. This method was developed and validated for use in bioavailability and bioequivalence studies.

Keywords: Validation; Acyclovir

1. Introduction

Acyclovir (ACV) or 9-(2-hydroxyethoxy)-methylguanine (Fig. 1) is a synthetic purinic nucleosidic analogue derived from guanine. This drug is structurally differentiated from guanine due to the presence of an acyclic lateral chain. This substance contains pK_a values of 2.27 and 9.95, and presents a water solubility of 1.3 mg/ml at 25 °C [1].

ACV is an important antiviral drug used exten-

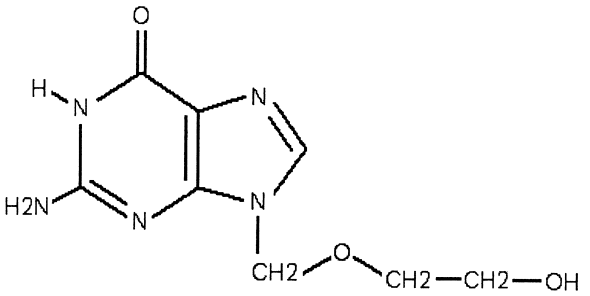

Fig. 1. Chemical structure of acyclovir.

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sively against infections caused by herpes viruses, especially herpes simplex and varicella zoster. Intravenous, oral and to a lesser extent topical ACV is commonly used in the treatment of herpes simplex (HSV) infection, with significant therapeutic results in genital herpes simplex as well as recurrent orofacial herpes simplex [2]. Topical ACV is the least effective in improving symptoms than are other formulations. Intravenous ACV is the accepted treatment of choice for herpes simplex encephalitis and has also shown positive results in the treatment of the severe complications associated with HSV infection during pregnancy and neonatal HSV infections. ACV is effective but controversial in the treatment of otherwise healthy individuals with varicella, although it has been recommended for the treatment of herpes zoster [1].

The pharmacokinetics parameters of ACV following oral administration generally are highly variable. Peak plasma concentrations of 0.46–0.83 or 0.63–1.21 mg/l after a single oral dose of 200 or 400 mg, respectively [1], and are generally obtained 1.5–2.5 h after administration [1,3,4]. ACV absorption in the gastrointestinal tract is slow, variable and incomplete [3]. The bioavailability of ACV is from 15 to 30% [3,4]. Approximately 80% of an oral dose is unabsorbed and is excreted with the feces [5]. The main excretory organ for ACV is the kidneys. The plasma half-life of oral ACV averages 3 h in adults with normal renal functioning. One main metabolite 9-carboxymethylguanine is formed in the liver at a rate of 8–14% relative to the ACV dose and is excreted via the kidneys [3]. The union to the plasmatic proteins occurs in a range of 9–33% [4,6].

Bioavailability studies with new, immediate-release oral preparation are essential for virustatics drugs for several reasons. Firstly, these products need to be systemically available to achieve their desired effects. Secondly, they can be used as an indication of reduced bioavailability, which could represent a risk to patients. Furthermore, bioavailability studies of newly developed tablet formulations are required in all instances due to the great inter-individual variability of the pharmacokinetics parameters of ACV, especially in the partial processes of absorption and elimination [5]. Additionally, the structural similarity of ACV with certain endogenous substances requires a very selective analytical method, which permits its quantification with sensitivity and for a wide range of concentrations.

Several studies have sought to determine drug content in pharmaceuticals, pharmacokinetics and optimal dosing of ACV and related compounds. Drug concentrations were measured either by immunological techniques or reversed-phase high-performance liquid chromatography (HPLC) [2].

Radioimmunoassays (RIA) [7] and enzyme linked immunoabsorbent assays (ELISA) [8] are very sensitive, but these methods are problematic due to their costly and time-consuming experimental procedures as well as and the need to develop antiserum or monoclonal antibodies [2]. Furthermore, RIA is also inconvenient due to the manipulation and disposal of radioisotopes.

Alternatively, reversed-phase HPLC techniques [9–21] have been largely used in the determination of ACV from a biological matrix.

Very sensitive HPLC techniques to determine ACV from plasma using fluorescence detection with very acid mobile phases [10,11] can be used to improve fluorescence. Although these methods increase sensitivity, the acidity of the mobile phase quickly damages the stationary phase resulting in a decrease in the useful column life. Other studies used techniques with thermostatized columns [10,11, 13,14], permitting a chromatographic process at a controlled temperature.

HPLC with UV detection [13–21] is a very common method used for the determination of ACV; however, some of these methods present a detection limit higher than 100 ng/ml [14,15,17,21], which is not sensitive enough for pharmacokinetic studies [9].

For the pretreatment of the samples, some studies have used plasma centrifugation and direct injection of the supernatant in the chromatographic column [11]. Other studies used deproteinized agents such as trichloroacetic acid [9,10,16–19], trifluoroacetic acid [15] and aluminum sulphate solution [14] after centrifugation and injection of the supernatant. However, these techniques do not permit an adequate purification of the extracts. Some published studies describe the use of solid-phase extraction [12,13,21], producing extracts free of impurities and with high rates of drug recovery. However, these techniques do not use an internal standard that could compensate the unadvertised loss during the extraction process.

This work describes a simple, rapid, sensitive and

precise analytical method to determine ACV in plasma using 5′-*N*-methylcarboxyamidoadenosine (MECA) as an internal standard with UV detection and extraction in a solid-phase. This method can be used in therapeutic drug monitoring and pharmacokinetics studies.

2. Experimental

2.1. Materials

All reagents used were of analytical grade and the solvents used were HPLC grade, Omnisolv (Gibbstown, USA.). The ACV USP was donated by Laboratories Pasteur (Concepción, Chile). Water was purified by Barndstead, Nanopure Infinity equipment. Plasma samples were obtained from a pool of normal human plasma, supplied by the clinical laboratory Diagnomed (Concepción, Chile).

2.2. Equipment

A liquid chromatograph (HPLC) Merck–Hitachi (Japan) with a quaternary Lachrom L-7000 Merck Hitachi pump (Japan), arrangement diode detector Lachrom-7450 Merck–Hitachi (Japan), Interface Lachrom L-7000 Merck–Hitachi (Japan), autosampler Lachrom L-7200 Merck–Hitachi (Japan), Lachrom D-7000 Multi HPLC System Manager Software Merck–Hitachi (Japan), Column Merck 50983 LiChrospher 100RP-18 (particle size 5 μm, 250×4 mm) and precolumn LiChrocart 4-4 LiChrospher 100 RP-18 (5 μm) Merck (Germany) was used. For solid-phase extraction (SPE), Waters Oasis HLB (1 ml, 30 mg) columns were used.

2.3. Chromatography

The composition of the mobile phase was 30 m*M* phosphate buffer, pH 2.6, containing sodium dodecyl sulfate (SDS) and acetonitrile 18%. The pH was measured by a pHmeter. Further, the mobile phase was filtered (0.2-μm filter) and degasified with ultrasonic. The flow was of 1.5 ml/min and the injection volume 100 μl. The ultraviolet detection ranged between 250 and 260 nm. All the analyses were performed at room temperature (22±2 °C).

2.4. Preparation of standards

A standard ACV solution of 400 μg/ml was prepared dissolving 20 mg of the standard drug, completed to 50 ml with nanopure water in a glass flask, obtaining a concentration of 120 μg/ml. Successive dilutions were made to obtain the following concentrations: 0.05, 0.30, 0.60, 1.20 and 1.80 μg/ml. Each volume was completed with acidified 5% acetonitrile solution at pH 3 with concentrated phosphoric acid in 25 ml glass flasks. To each 1 ml of solution, 120 μg/ml of MECA were added, obtaining a concentration of 4.8 μg/ml of the internal standard. All the solutions were stored in a refrigerator at 4 °C during their manipulation.

2.5. Preparation of the samples

Plasma samples charged with a known quantity of ACV and MECA were submitted to extraction in a solid-phase, with Waters Oasis HLB (1 ml, 30 mg) columns.

The columns were placed in a vacuum distributor equipment of 12 points connected to a vacuum pump. The columns were conditioned with 1000 μl of methanol, followed by 1000 μl of nanopure water then the mixture was added to the column. When the samples were passed through the columns, the ACV and MECA were retained. To eliminate impurities, the columns were then washed with 1000 μl of nanopure water. ACV and MECA were eluted since the column with 750 μl of 5% acetonitrile solution acidified at pH 3 with concentrated phosphoric acid. One hundred μl of the eluted solution were injected in the chromatographic column.

2.6. Validation

The chromatographic method was validated on two different days, to determine the linearity, sensitivity, precision and accuracy of the present HPLC method.

Linearity was determined by a calibration curve with standard ACV and MECA solutions in the range of 0.05–1.80 μg/ml, with $n=10$, where the slope was calculated using the ACV concentration versus the areas ratio between ACV and MECA. The interpolation of the ratio areas ACV/MECA obtained

in the plasma samples in the standard calibration curve permitted quantification.

Precision was determined by the elaboration of three standard calibration curves, two from the same day (intra-day precision) and the third one from a different day (inter-day precision). Accuracy was determined by the recuperation percentage obtained when the samples charged with ACV were analyzed at concentrations of 0.05, 0.80 and 1.73 μg/ml, all with a constant concentration of 4.80 μg/ml of MECA, with $n=3$. Sensitivity was determined by the detection limit (3 times the value of the noise signal) and the quantification limit (10 times the value of the noise signal).

3. Results and discussion

3.1. Chromatography

ACV analysis presents many difficulties because it is a nucleoside analogue of guanine, which increases the possibility of endogenous interference. To increase selectivity and specificity of the technique, we included an ionic matching reagent, Sodium dodecylsulphate (SDS 5 m*M*), as a contrary ion to the mobile phase composition. We used acetonitrile 18% in a tampon medium with phosphate buffer at pH 2.6 as an organic modifier. As a result, the standard solutions of ACV and MECA moved, and symmetric well-defined peaks were obtained, with an average retention time for ACV of 5.0 min and 11.2 min for MECA (Fig. 2).

No interfering peaks were observed in the chromatographs of plasma without drug content (Fig. 3). In the samples of plasma charged with ACV and MECA, chromatographs with well-resolved and symmetric peaks were observed (Fig. 4). Additionally, no interferences for ACV or for MECA were observed.

The diode arrangement detector permitted better visualization of the absence of endogenous interferences in the sample matrix because it provided information not only for a discrete wavelength, but also for the entire absorption spectrum (200–400 nm).

Furthermore, the duration of the chromatogram

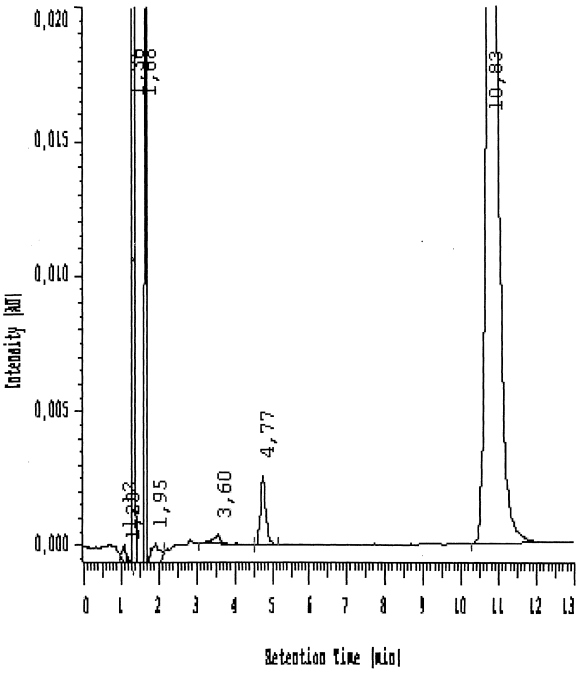

Fig. 2. Chromatograph of ACV/MECA standard solution 0.152/4.800 μg/ml.

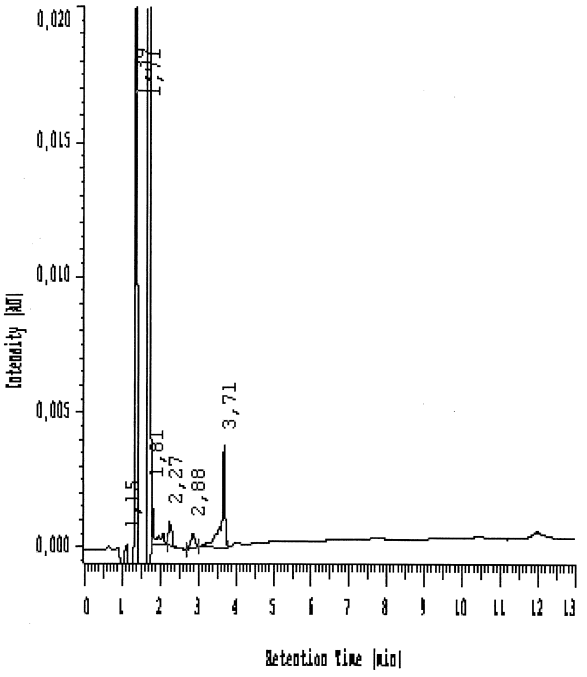

Fig. 3. Chromatograph of the plasma sample without ACV content.

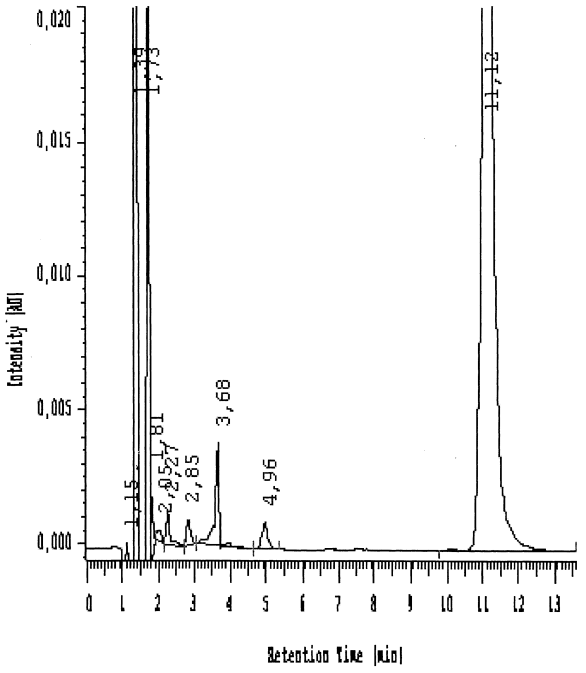

Fig. 4. Chromatograph of the plasma sample with standard ACV/MECA 0.053/4.800 μg/ml content.

was 13 min, which permitted the analysis of a large number of samples in a short period of time.

3.2. Preparation of the samples

In preparing the samples, we tested several methods to improve the sensitivity while at the same time reducing the time spent in performing the test.

For the pretreatment of the samples, we tested deproteinized agents with supernatant injection as used in other studies [9,10,14–19]; however, the extracts obtained presented a high level of impurities. Columns C_{18} [12,18] were used with better results in obtaining extracts free of interferences and with good drug recuperation (±80%). However, due to the hydrophobic nature of the C_{18} filling, the sample needs to be conditioned taking care not to dry it, limiting significantly the number of samples to be analyzed simultaneously.

Taking into account the low union of ACV to proteins, ultrafiltration [20] was also tested. But the extracts obtained were not free of impurities and there was low drug recovery (less than 50%).

Finally, we tested solid-phase extraction using Waters Oasis HLB columns containing polymeric filling for reversed-phase with hydrophilic and lipophilic characteristics. The Oasis filling is hydrophilic and thus can be maintained damp with water, maintaining their retention capacity and good recuperation (higher than 90%), even when the filling is dry. Furthermore, this filling does not contain silanol groups, avoiding the problem of ionic interchange interactions between residual silanols in C_{18} cartridges and ACV [21]. Likewise, the use of these cartridges permits a more precise and less tedious process than with the conventional C_{18}, allowing simultaneous analysis of a higher number of samples.

The elution solution used was an acetonitrile concentration of 5%, intended to achieve the maximum elution of ACV with minimum interferences. The elution medium was acidified to favor the most polar form of the molecule, which is less joined to the polymer of the filling column and hence the ACV is more easily dragged by the eluent. Similarly, when the elution is performed with 750 μl of 5% acetonitrile solution acidified at pH 3 with phosphoric acid, virtually all of the ACV and MECA was eluted without dragging undesirable substances that could provoke interfering peaks.

3.3. Linearity

The standard calibration curve was linear ranging between 0.05 and 1.80 μg/ml, with a correlation coefficient (r^2) of 0.9999. The linearity indicator for the standard calibration curve of ACV, t_R, was of 761.5 and t_{table} was 2.002 with 58 grades of liberty ($n=10$). Similarly, t_R is higher than t_{table}, indicating that a significant linear correlation exists, with the probability level of 0.05 for the selected concentration range.

3.4. Precision

Intra- and inter-day repetition determined the precision method. The statistical indicator for preci-

sion method F_c in intra- and inter-day was lower than $F_{theoretical}$, and was not significant for the probability level of 0.05. No evidence favoring the inequality of the straight regression lines was observed. The coefficients of variation (C.V.) of ACV in the three straight regression lines ranged between 0.29 and 4.02, indicating a low variability between the values obtained for each concentration.

3.5. Accuracy

The plasma samples charged in three concentration levels: low (0.05 μg/ml), medium (0.80 μg/ml) and high (1.80 μg/ml), recovering between 94.7 and 109.7% with a C.V. ranging between 1.6 and 5.2% as shown in Table 1. The recuperation percentage of MECA was 99.6% with a C.V. of 3.5.

The recovery percentages obtained did not differ from the real value in more than±10%, while the C.V. were lower than 5.2%. In both cases the values obtained were lower than the limits required for biological samples, ± 15% for the recuperation and 15% for C.V. [22].

3.6. Sensitivity

The detection limit was of 0.006 μg/ml and the quantification limit of 0.020 μg/ml. This method was sufficiently sensitive, with a quantification limit lower than the minimum concentration recommended (0.04 μg/ml) for plasma samples obtained after the administration of a 200-mg dose of ACV.

4. Conclusion

Of all the methods evaluated for ACV extraction from plasma samples, the solid-phase extraction with Waters Oasis HLB cartridges is the technique that yielded the best results. This technique eliminated endogenous interferences, provided high and precise recuperations, and permitted the simultaneous analysis of a large number of samples in a short period of time. The inclusion of an internal standard allowed for the compensating losses that would have been produced during the extraction process. Likewise, using HPLC with UV detection it was possible to develop a sensitive, selective method that is at the same time simple and rapid for the determination of ACV in plasma. This method provides a good alternative for monitoring and pharmacokinetics studies in a wide range of concentrations. The proposed technique should be tested in future bioavailability and bioequivalence studies of ACV.

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Table 1
Accuracy and precision of plasma samples charged with acyclovir and MECA

ACV conc. (μg/ml)	Accuracy (% recuperation) (Mean, n=3)		Precision % (C.V.) (n=3)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.05	Series 1: 109.7		4.2	
		Series 3: 107.7		5.2
	Series 2: 105.3		3	
0.8	Series 1: 96.7		1.8	
		Series 3: 99.8		1.9
	Series 2: 98.9		2.7	
1.73	Series 1: 94.7		2.5	
		Series 3: 96.3		1.6
	Series 2: 96.0		1.8	

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