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Development of a sensitive method for the determination of ganciclovir by reversed-phase high-performance liquid chromatography

M. Merodio[a], M.A. Campanero[b], T. Mirshahi[c], M. Mirshahi[c], J.M. Irache[a,*]

[a]*Centro Galénico, Universidad de Navarra, Ap. 177, 31080 Pamplona, Spain*

[b]*Servicio de Farmacología Clínica, Clínica Universitaria de Navarra, 31080 Pamplona, Spain*

[c]*INSERM XR86, Université de Paris VI, 75006 Paris, France*

Abstract

Ganciclovir is a nucleoside analogue widely used in the treatment of cytomegalovirus infections, which affects mainly immunocompromised patients. Recently, new pharmaceutical dosage forms based on the use of albumin nanoparticles have been developed for improving the efficacy of this drug. The aim of this study was to develop an analytical HPLC method for the determination of ganciclovir in both pharmaceuticals (i.e. albumin nanoparticles) and biological medium samples. The chromatography was performed on a reversed-phase encapped column (LiChrospher Select B C_8) with a mobile phase consisting of acetonitrile in 0.05 *M* ammonium acetate (pH 6.5; 2: 98, v/v). Acyclovir was used as internal standard and the detection wavelength was 254 nm. The limit of quantitation of ganciclovir was 50 ng/ml and the average recoveries over a concentration range of 0.05–10 μg/ml ranged from 98 to 102%. Precision did not exceed 5%. In summary, this assay is a selective, sensitive and reproducible method for the determination of the ganciclovir in albumin nanoparticles. It can be successfully applied to the estimation of the ganciclovir uptake by cultured human corneal fibroblasts.

Keywords: Pharmaceutical analysis; Ganciclovir; Acyclovir; Nucleoside analogues; Albumin

1. Introduction

Ganciclovir, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine (Fig. 1A), is a synthetic nucleoside analogue closely related to acyclovir (Fig. 1B). This drug exhibits antiviral activity against human herpes viruses at relatively low inhibitory concentrations [1,2]. Moreover, in a number of clinical studies, it has been proved effective against cytomegalovirus (CMV) in immunocompromised patients, mainly in those with the acquired immunodeficiency syndrome (AIDS), congenital immunodeficiency or in individuals following organ transplantation [3,4]. These viral infections are one of the main causes of morbidity and mortality. In these patients, ganciclovir prolongs the time of the progression of cytomegalovirus retinitis and, therefore, maintenance therapy is necessary to control the disease.

For the treatment of cytomegalovirus retinitis, intravitreal administration has been approved as a safe and well tolerated alternative for providing concentrations of ganciclovir within the therapeutic

*Corresponding author. Tel.: +33-948-425-600; fax: +33-948-425-649.

E-mail address: jmirache@unav.es (J.M. Irache)

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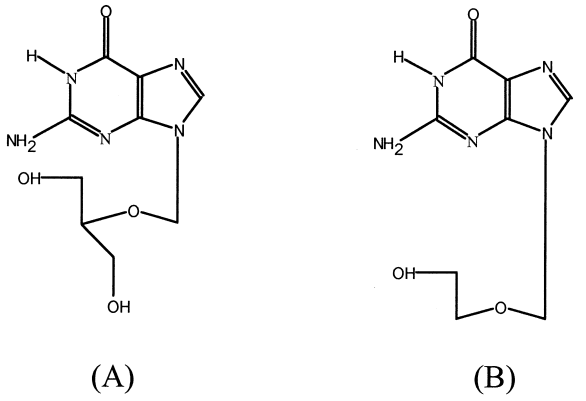

Fig. 1. Chemical structures of ganciclovir (A) and acyclovir (B) used as internal standard (I.S.).

range in the eye, decreasing the incidence of systemic adverse effects [5]. In this context, we have recently developed new ganciclovir delivery systems based on albumin nanoparticles. These pharmaceutical dosage forms are able to sustain continuous drug release and, therefore, reduce the intravitreal administration frequency.

A number of different analytical studies have been done to determine drug content in pharmaceuticals, pharmacokinetics and optimal dosing of ganciclovir. In these studies, ganciclovir concentrations were determined by immunological techniques [6–8] or reversed-phase high-performance liquid chromatography [9–12].

While radioimmunoassay (RIA) or enzyme-linked immunosorbent assays are very sensitive, they have disadvantages such as the sample cost and the great number of slow steps of the analytical process [6].

Alternatively, chromatographic techniques have also been widely used for the analysis of ganciclovir in drug products and biological samples. Though adequate for the task, these procedures are not suitable for large-scale pharmaceutical analysis, since they show long turnaround times [9,10] and, sometimes, analytical conditions which can limit column life. Some methods included ion-pair agents [12,13] or organic modifiers (i.e. triethylamine) [14] in the mobile phase composition. These compounds can induce the aggregation and the precipitation of polymers or macromolecules usually employed in the preparation of nanoparticulate dosage forms inside the columns, resulting in the reduction in the column separative capacity, loss of peak quality and dramatic increase in the back pressure.

The aim of this study was to develop an analytical HPLC method for the simple, sensitive, specific and rapid determination of ganciclovir in both pharmaceuticals (i.e. albumin nanoparticles) and biological medium samples.

2. Experimental

2.1. Chemicals and reagents

Ganciclovir (Cymevene®) was obtained from Roche (Madrid, Spain) and acyclovir was provided by Alonga (Madrid, Spain). Ammonium acetate, acetonitrile and all solvents and reagents used were of HPLC analytical grade and were supplied by Merck (Darmstadt, Germany).

2.2. Calibration standards

A stock solution of ganciclovir (1 mg/ml) was prepared by dissolving 54.6 mg in water. A second stock solution was also prepared by diluting in water ten times the first stock solution (0.1 mg/ml). Working-standard solutions were prepared in water from the stocks solutions. The concentration range of ganciclovir for the standard curve samples was between 50 and 10 000 ng/ml.

On the other hand, 55.5 mg acyclovir sodium was dissolved in 50 ml of water to obtain a stock solution of 1 mg/ml. The working standard was prepared in water from the stock solution at 100 μg/ml. Standard curve samples were prepared by adding 50 μl of the internal standard (I.S.) working standard solution to 1 ml of the ganciclovir working standard samples.

All solutions could be kept at room temperature for up to 12 days.

2.3. Sample preparation

2.3.1. Bovine serum albumin nanoparticles

Albumin nanoparticles were prepared by means of a coacervation technique and chemical cross-linking with glutaraldehyde. According to the drug incorporation two different procedures were assayed. The

first one (also called Model A) was obtained by the addition of ethanol dropwise to an albumin (2%, w/v) aqueous solution. These coacervates were hardened with glutaraldehyde for 2 h and the resulting unloaded nanoparticles concentrated by evaporation under reduced pressure. Then, model A nanoparticles were obtained by incubation with different amounts of ganciclovir for 2 h.

The second one (called Model C) was prepared by the incubation of an aqueous albumin solution (2%, w/v) with the drug and the cross-linking agent. Then, the pH of the solution was adjusted to 5.5 with 1 *M* HCl prior to the addition of ethanol and the resulting nanoparticles were concentrated by evaporation under reduced pressure.

Finally, the different ganciclovir-loaded albumin nanoparticle batches were purified by centrifugation at 19 500 rpm, for 45 min (Rotor SS-34; Sorvall RC5-plus). Then, the recovered supernatants were diluted to 100 ml in water, spiked with I.S. and the amount of ganciclovir evaluated by injection of 1 μl of this solution into the HPLC system.

2.3.2. Determination of ganciclovir uptake by cells in culture

Human corneal fibroblasts were cultured in RPMI-1640 medium (Gibco, Paris, France) with glutamax, supplemented with 10% heat-inactivated foetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco), and incubated for 96 h with ganciclovir-loaded albumin nanoparticles.

After removing the supernatants, cells were detached by the harvesting addition of trypsin-EDTA (2.5%) and digested with triton (1%). After centrifugation at 17 000 rpm for 10 min, the supernatants were diluted with water and the samples stored at −20°C till chromatographic determination. The analysis was carried out in the same way as aforementioned (injection volume: 10 μl).

2.4. Instrumentation and chromatographic conditions

The instrument was equipped with a Hewlett-Packard (HP) 1050 quaternary pump; a HP 1050 autosampler and a HP 1050 diode-array detector set at a wavelength of 254 nm (λ_{max}). Software packages used were Chemstation 3D (Hewlett-Packard, Waldbronn, Germany) for chromatographic analysis and SPSS for statistic analysis.

The samples were chomatographed on a reversed-phase 250×4 mm C_8 LiChrospher Select B column (5 μm) provided by Merck. A 4×4 mm precolumn of the same material was also used. The mobile phase consisted of 2% acetonitrile in 0.05 *M* ammonium acetate (pH≈6.5). Mobile phase, filtered and degassed before use, was pumped at 1 ml/min flow-rate. The column was thermostated at 40°C. Under these experimental conditions the run time was 8 min.

For robustness studies, different reversed-phase columns, such as Hypersil ODS (3 μm particle size; 10×0.46 cm; Teknokroma) and Spherisorb C_{18} (10×0.46 cm; Teknokroma) were used. A 1×0.46 cm precolumn of the respectively same materials was used in each case. Similarly, the influence of the mobile phase (percentage of acetonitrile ranged from 1 to 5%) and column temperature (25°C or 30°C) on the analytical procedure was also evaluated.

2.5. Application of the method

This HPLC method was applied to the determination of both the ganciclovir (GCV) content in nanoparticulate dosage forms and the amount of drug taken up by cultured cells.

On the one hand, different ganciclovir-loaded albumin nanoparticle batches were prepared to study the influence of the ganciclovir bulk concentration on the payload. The drug loading (payload) was calculated as follows:

$$\text{Drug loading} = \frac{\text{amount of GCV in nanoparticles (μg)}}{\text{albumin nanoparticle yield (mg)}} \cdot 100 \quad (1)$$

The amount of ganciclovir in nanoparticles was determined as the difference between the initial added drug and the amount recovered in the supernatants after sample centrifugation of samples. The nanoparticle yield was determined by digesting the albumin nanoparticles with 1 *M* NaOH at room temperature for 24 h. The absorbance of this solution was then measured in a spectrophotometer at 280 nm (Diode-Array HP 8452, Hewlett-Packard) and com-

pared with the absorbance value obtained after digestion of a control albumin solution.

On the other hand, the capacity of albumin nanoparticles as pharmaceutical systems for carrying ganciclovir to human corneal fibroblasts was also studied. Corneal fibroblasts were seeded in tissue culture plates and incubated with ganciclovir-loaded nanoparticles as previously described. Then, the amount of ganciclovir taken up by cultured cells was evaluated after drug extraction from the inner of cells.

3. Results

3.1. Validation of the method

3.1.1. Chromatography

The analytical peaks of ganciclovir (GCV) and acyclovir (I.S.) were well resolved from one another and typical matrix components such as waste products resulting during the preparation of nanoparticles or cell components extracted with the drug (Fig. 2).

3.1.2. Selectivity of the assay

The selectivity of the assay was determined by the individual analysis of blank samples from cell culture media, with and without internal standard. Under these chromatographic conditions, no endogenous sources of interference were observed in the medium and the resolution between ganciclovir and I.S. was satisfactory.

3.1.3. Sensitivity of the assay

Detection and quantification limits (LOD and LOQ, respectively) of the HPLC assay was determined by the analysis of the peak baseline noise in five blank samples. Thus, LOD, determined as 3 times the variation in measured response, was calculated to be 10 ng/ml. Similarly, the limit of quantification, estimated as 10 times the variation in the measured response, was calculated to be 50 ng/ml. This LOQ was confirmed, in separate experiments using calibrators with a nominal concentration of 50 ng/ml, with aqueous solutions and mediums from cell cultures. The mean assay result was 48.96 ± 1.54 ng/ml ($n = 6$) with a relative standard deviation of 3.14%.

3.1.4. Extraction efficiency

Absolute recovery was investigated by compared analysis of extracted samples both from the supernatants of the nanoparticles ad the cell culture mediums versus directly injected standards at concentrations of 50, 500 and 10000 ng ganciclovir per ml ($n = 9$). Ganciclovir recoveries ranged from $97.9 \pm 1.78\%$ to $102.1 \pm 2.62\%$.

3.1.5. Linearity of the assay

Linearity was determined by plotting a standard curve from the ratio between the ganciclovir peak area to the acyclovir peak area versus the corresponding drug concentrations in water. Standard curves were found to be linear on three different days over the range 50–10000 ng/ml. Linear regression analysis showed correlation coefficients greater than 0.999 in all curves. What's more, the accuracy of the calibration curves was also determined (Table 1).

Moreover, a linearity test, by comparing calculated standard points to the nominal ones, was carried out on calibration curves in order to confirm the linearity and to test the quality of the fitting [16]. The assays exhibited linearity ($r > 0.999$), with a slope near to the unit (calculated t value was 457.712) and an intercept (calculated t value was 0.05) not statistically different from zero. The critical t value was 0.49 ($P = 0.01$).

3.1.6. Accuracy of the assay

Accuracy of the assay was defined as the percentage of the systematic error, which was calculated as the agreement between the measured value and the true value as follows:

$$\text{Accuracy} \frac{\text{True value} - \text{Measured value}}{\text{True value}} \cdot 100 \qquad (2)$$

Accuracy values in intra-day variation studies at low, medium and high ganciclovir concentrations were always within acceptable limits ($< 15\%$) at all concentrations (see Table 2) [17].

3.1.7. Precision of the method

In this work, precision of the method was tested on both the within-day and between-day reproducibility in the assay. Within-day variability of the assay method was determined by repeated analysis of four

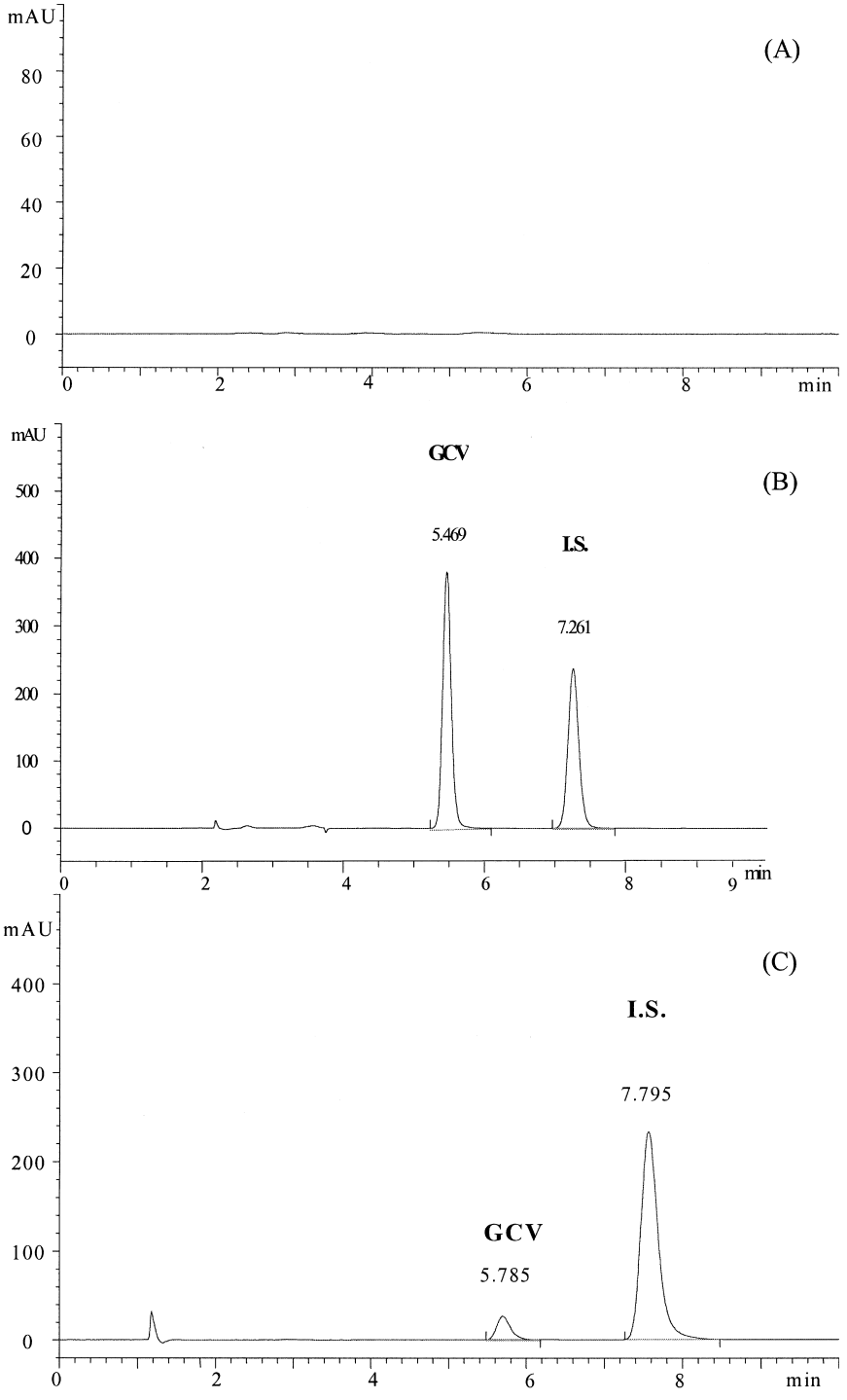

Fig. 2. Chromatograms resulting from the analysis of a blank medium from cultured cells (A), a supernatant obtained during the purification step of ganciclovir-loaded albumin nanoparticles (B), and a sample from the determination of ganciclovir taken up by cultured cells after incubation for 96 h with ganciclovir loaded nanoparticles. I.S. = Acyclovir, GCV = ganciclovir.

Table 1
Statistical evaluation of the ganciclovir analysis results in standard curves over 3 days. The calculated calibration curve for ganciclovir aqueous solution was typically $y=0.000177x+0.000734$ ($n=6$); ($r=0.9999$)

Accuracy ($n=9$)		
Concentration added (ng ml^{-1})	Concentration found (mean±SD) (ng ml^{-1})	Relative error (%)
50	45.22±5.73	−2.00
100	95.93±7.85	−4.07
250	235.12 ±6.84	−5.95
500	510.45±13.13	2.09
1000	1 034.72±3.77	3.47
5000	4 884.15±11.63	−2.32
10000	10 043.40±14.48	0.43

Table 2
Accuracy of the method, expressed as relative error in %, for determining ganciclovir concentrations

Accuracy ($n=5$)		
Concentration added (ng ml^{-1})	Concentration found (mean±SD) (ng ml^{-1})	Relative error (%)
50	48.18±1.23	−3.77
500	518.42±0.36	3.68
5000	4885.81±2.04	−2.28

quality control samples at low, medium and high concentrations on the same day. The results are shown in Table 3. These data clearly indicate that the assay method is reproducible within the same day. Similarly, between-day variability was determined by repeated analysis of four quality control samples at low, medium and high concentrations on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided into aliquots that were stored at −20°C until analysis. The results summarised in Table 3 also indicate that the HPLC assay was reproducible between different days [17].

3.1.8. Robustness

For the robustness study, different analytical columns (Hypersil ODS and Spherisorb C_{18}) and guard columns were successfully used with no significant variations in the chromatography results. If the temperature of the column was decreased to 30°C or

Table 3
Between and within-day variability of the HPLC method for determining ganciclovir concentrations

Concentration added (ng ml^{-1})	Between-day variability ($n=5$)		Within-day variability ($n=5$)	
	Concentration found (mean±SD) (ng ml^{-1})	RSD (%)	Concentration found (mean±SD) (ng ml^{-1})	RSD (%)
50	48.96±1.54	3.14	48.18±1.23	2.55
100	101.96±0.96	0.94	95.93±7.85	1.00
500	501.60±13.69	2.73	518.42±0.36	0.07
5000	4878.45±11.73	0.24	4885.81±2.04	0.04

25°C, the elution times were not modified. A higher percentage of acetonitrile (5%) in the mobile phase decreased the ganciclovir and acyclovir retention times (3.5 and 4.3 min, respectively, with 5% acetonitrile and 5.2 and 6.9 min with 1%), eluting both peaks more closely. Variance in the flow-rate resulted in changes in the retention times, prolonging the chromatogram time.

Nevertheless, no single parameter, extended to the specified limits, resulted in a dramatic adverse effect on the system suitability.

3.1.9. Stability

Stability of ganciclovir standard solutions stored at 4°C was demonstrated for 1 month. Ganciclovir concentrations in mediums from cell cultures were stable when stored at −20°C for at least 3 months. In any case, no changes in the drug concentration were detected when the extracted samples were stored at room temperature for 48 h or 37°C for 1 month.

3.2. Application of the method

The reported method was used for the determination of ganciclovir content in albumin nanoparticles and its incorporation to cultured cells.

On the one hand, the drug loading was calculated by means of Eq. (1) (see Experimental section). This parameter was plotted against the ratio between the initial amount of GCV and the initial amount of protein added to ganciclovir-loaded albumin nanoparticles. The results are reported in Fig. 3. It was clear that the payload of ganciclovir associated with nanoparticles increased with the concentration of the drug. Furthermore, the preparation of albumin nanoparticles following the model C procedure enabled us to improve their ganciclovir drug loading capacity. However, the loading capacity of GCV to model C albumin nanoparticles was limited at ratios greater than 250 μg drug/mg protein. Under these conditions, the solubility of ganciclovir was compromised.

On the other hand, Fig. 4 shows the ganciclovir uptake by human corneal fibroblasts after incubation with either a drug aqueous solution or albumin nanoparticles. In all cases, the uptake increases with the concentration of the drug. However, model A

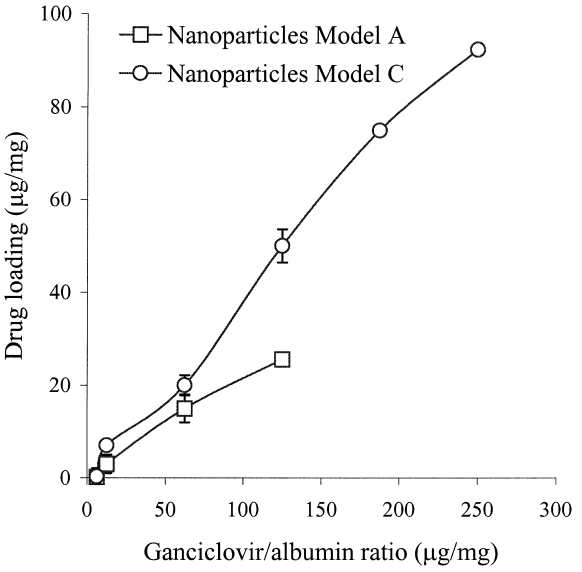

Fig. 3. Application of the method: Influence of the ganciclovir/albumin ratio on its drug loading (μg drug/mg nanoparticles). Experiments were performed with an initial amount of albumin of about 80 mg and the typical nanoparticle yield was about 50±2.1% for model A and 60±3.2% for model C.

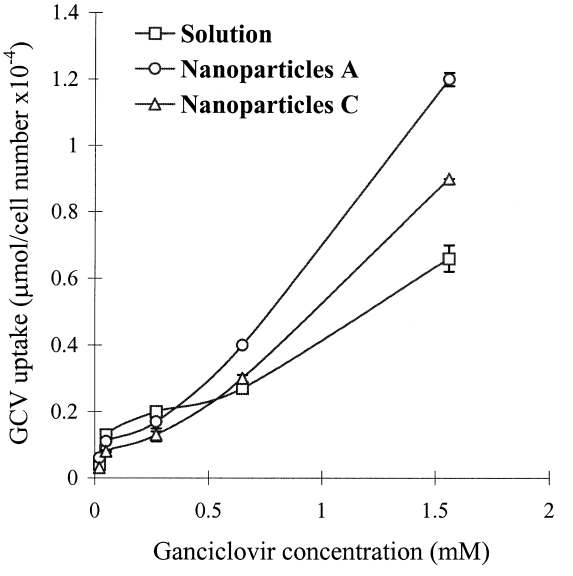

Fig. 4. Application of the method: Ganciclovir taken up by cultured human corneal fibroblasts. Number of cells = 6×10^4 cells/cm^2.

nanoparticles provided higher levels of ganciclovir uptake by cells than model C nanoparticles. Moreover, the determination of ganciclovir was selective, without interferences of other cellular components.

4. Discussion and conclusions

The quantitation of antiviral agents in biological mediums is quite difficult because these drugs show a chemical structure which is quite similar to a number of endogenous substances. This fact makes analysis difficult and requires the use of highly selective analytical methodology, such as reversed-phase liquid chromatography.

Therefore, the analysis of ganciclovir by HPLC shows more difficulties, since it is a nucleoside analogue of guanine. Moreover, ganciclovir is a strongly polar molecule, so is slightly retained by apolar stationary phases ($k'<1$) such as C_{18} reversed-phase columns. In this context, some authors have proposed the use of ion pair agents in the mobile phase composition to increase the selectivity and specificity of the technique, when biological samples (i.e. cell extracts) have to be analysed. These agents decrease ganciclovir polarity, by a chemical reaction with one of its polar groups, increasing its k' value to 3, with the help of an appropriate selection of the mobile phase pH value. Although ion-pair reagents have been extensively used in the development of chromatographic methods, it is well known that they can cause a rapid deterioration of the column, ghost peaks and baseline disturbances [15].

At the beginning of our research, we attempted to use the analytical conditions included in the published methods [14]. However, the results obtained in our laboratory with these reported methods showed a high variability and a low degree of selectivity when biological samples were analysed. Moreover, the lifetime of the columns was very short under these chromatographic conditions when supernatants from nanoparticle suspensions were injected. This last problem was probably due to an interaction between albumin residues and ion-pair agents resulting in the aggregation and precipitation of the complex.

In order to solve these problems we included ammonium acetate in the mobile phase to avoid the use of an ion pair agent or other organic modifiers and a normal-phase HPLC column was chosen. Ammonium acetate is a good agent for improving the ganciclovir solubility in solvents usually employed as mobile phases in normal-phase HPLC methods. Moreover, it is interesting to note that this chemical also has many properties which are particularly interesting for use as a general purpose buffer [13]. The most significant are the following: low toxicity and price, easy availability, good buffering capacity in the chosen pH range and a good agent for providing greatly improved separations without column deterioration.

In addition, we have replaced the C_{18} reversed-phase column with a slightly more polar encapped stationary phase; the LiChrospher C_8 60 RP-select B column. Under these conditions the selectivity of the chromatographic method was optimum to allow us the quantification of ganciclovir in the mediums described above. In summary, this assay is a selective, sensitive and reproducible method for the separation and determination of ganciclovir in pharmaceuticals from the rest of excipients and materials used to prepare the dosage form (i.e. macromolecules). Similarly, it can be successfully applied to the calculation of the ganciclovir content in biological samples. The precision and accuracy of the proposed method are within acceptable ranges and the quantitation limit is as low as 50 ng/ml. Finally, the assay is robust but variance in the percentage of acetonitrile in the mobile phase should be specially monitored.

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