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Development and validation of a sensitive method for the determination of ganciclovir in human plasma samples by reversed-phase high-performance liquid chromatography

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Abstract

A rapid, sensitive, specific liquid chromatographic method has been developed for the determination of therapeutic levels of ganciclovir in human plasma. Plasma (1 ml) and acyclovir (I.S.) were treated with 50% trichloroacetic acid. The supernatant was neutralized with 2 *M* NaOH and purified with chloroform. The aqueous phase (80 μl) was analyzed by a 3-μm Hypersil ODS C_{18} column with 0.04 *M* triethylamine–0.1 *M* sodium dihydrogen phosphate monohydrate as the mobile phase (1 ml/min) and ultraviolet detection at 254 nm. Calibration was linear from 50 to 10 000 ng/ml. Intra- and inter-day C.V. did no exceed 6.65%. The detection limit was about 10 ng/ml. © 1998 Elsevier Science B.V.

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1. Introduction

Ganciclovir, (9-(1,3-dihydroxy-2-propoxymethyl) guanine (Fig. 1), is a potent antiviral drug that has shown activity against herpes viruses, varicella-zoster virus, cytomegalovirus and Epstein-Barr virus. It shows, after phosphorylation to ganciclovir triphospate, greater affinity for DNA polymerase. This affinity is selective, inhibiting only the viral enzyme [1]. Ganciclovir is the first-election therapy for cytomegalovirus in immunocompromised patients, like immunocompromised organ transplant patients and patients with acquired immunodeficiency syndrome (AIDS) [2]. In AIDS patients, ganciclovir is effective in AIDS-related gastrointestinal and pulmonary infection by cytomegalovirus.

A few methodologies which include radioimmunoassay (RIA) and enzyme-linked inmunosorbent assay have been reported for the quantification of this antiviral drug in biological fluids. These tech-

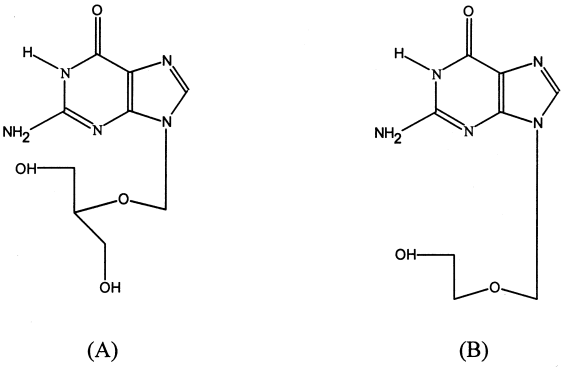

Fig. 1. Structures of (A) ganciclovir, and (B) acyclovir (I.S.).

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niques have significant disadvantages, due to the large number of steps and the lengthy time required to obtain final quantitative results [3].

Some high-performance liquid chromatographic (HPLC) methods have been described for the analysis of ganciclovir in plasma. These methods have used ion-pairing agents [3,4], gradient elution [5], electrochemical detection [6] and ion-exchange chromatography [6] to resolve ganciclovir. In addition, these procedures have long turnaround times, low sensitivity and a limited column life.

One method described in the bibliography [7] showed adequate sensitivity and extended column life (3000 samples) to quantitate ganciclovir in pharmacokinetic samples. However, it failed to incorporate an internal standard. The internal standard corrects inadvertent changes in drug concentration incurred during extraction, dilution or concentration of plasma homogenates. Other methods [8,9] described in the bibliography, which incorporated internal standard, showed adequate selectivity and short times to quantitate ganciclovir in pharmacokinetic samples. However they appeared to have low sensitivity and medium column lifetime (600 samples).

In this work, we have developed and validated a sensitive and precise analytical method for the determination of ganciclovir, incorporating an internal standard, which can be applied for the determination of ganciclovir concentrations in plasma. This method can be applied to therapeutic drug monitoring and pharmacokinetic studies in normal patients, patients with several impaired renal function, and heart, hepatic and renal transplant patients.

2. Experimental

2.1. Chemicals and reagents

All solvents and reagents were HPLC and analytical grade respectively, and were obtained commercially from Merck (Darmstadt, Germany). Syntex (Paris, France) provided Ganciclovir. Acyclovir (I.S.) was provided by Alonga (Madrid, Spain).

2.2. Apparatus and chromatographic conditions

A Hewlett Packard (Waldbronn, Germany) HPLC apparatus was used. The instrument was equipped with a HP 1050 quaternary pump; a HP 1050 autosampler and a HP 1050 diode-array detector set at wavelength of 254 nm. Software packages used for this work were Chemstation 3D (Hewlett Packard, Waldbronn, Germany) for chromatographic analysis and SPSS for statistic analysis. A HP VECTRA VL2 4/66 computer (Hewlett Packard, Waldbronn, Germany) performed these packages.

Separations were achieved on a reversed-phase Hypersil ODS column (3 μm, 100×4.6 mm I.D.) provided by Teknokroma (Barcelona, Spain). A 10× 4.6 mm precolumn was used. The mobile phase consisted of 0.1 *M* sodium dihydrogen phosphate monohydrate and 0.04 *M* triethylamine. Mobile phase, filtered and degassed before use, was pumped at 1 ml/min flow-rate at 40°C. The pH of the mobile phase was 6.6.

2.3. Standard solutions and samples

A stock solution of ganciclovir (1 mg/ml) was prepared by dissolving 50 mg of ganciclovir salt in water. Working standards were prepared in water from stock solution in the range 1–200 μg/ml by the sequential dilution method. A stock solution of acyclovir (I.S.) (1 mg/ml) was prepared by dissolving 50 mg of acyclovir salt standard in 50 ml of water. Working standards were prepared in water from stock solution at 100 μg/ml.

Stock solutions and working-standard solutions of ganciclovir and acyclovir were stored at 4°C. Standard curve samples of the biological fluids were prepared by spiking 50 μl of each standard solution of ganciclovir and 50 μl of standard solution of acyclovir (I.S.) into 1 g of blank samples. The concentration range for the standard curve samples was 50 to 10000 ng/ml.

2.4. Extraction and isolation procedures

Plasma samples (1 g) were spiked with internal standard (acyclovir) and deproteinized with 100 μl of 50% trichloroacetic acid. After shaking (30 s), the

deproteinized samples were centrifuged at 2000 *g* for 10 min. Then, the supernatant was removed to a new tube and neutralized with 2 *M* sodium hydroxide (50 μl). The mixture was vortexed during 10 s and then extracted with 5 ml of chloroform. The contents of these tubes were stirred vigorously for 30 s, and centrifuged at 3000 *g* for 5 min. Aliquots of the aqueous phase (400 μl) were mixed with 40 μl of 1 *M* sodium dihydrogen phosphate monohydrated–0.4 *M* triethylamine solution, and 80 μl were injected onto the HPLC system.

2.5. Application of the method

One patient was receiving an intravenous infusion of ganciclovir (Cymevene, Syntex) at 5 mg/kg dose for 45 min. Blood samples obtained at different times (−10, 15, 30, 45, 47, 60, 75, 105, 165, 285, 405, 525 min) were centrifuged for 10 min at 3000 *g* and the plasma was stored at −20°C until analysis.

3. Results

3.1. Detection wavelength

Solutions of ganciclovir exhibited two absorption peaks in the ultraviolet-visible spectrum (254 and 275 nm). The absorption at 254 nm was higher. Solutions of acyclovir exhibited two absorption maxima at same wavelength than ganciclovir. At 254 nm the spectrophotometric response from the detector for the internal standard concentration was reproducible, therefore this wavelength was selected.

3.2. Chromatography

Ganciclovir and acyclovir (I.S.) had a retention time of 4.0 and 5.1 min, respectively, in the chromatographic conditions described. Fig. 2 shows the chromatograms from the analysis of blank human plasma samples, plasma spiked with 50 ng/ml of ganciclovir and I.S., and the plasma sample obtained at 2 min post-infusion from a subject receiving a 5 mg/kg intravenous infusion-dose of ganciclovir, respectively. With the sample preparation procedure above, the blank chromatogram contained no significant peaks at the retention time corresponding to ganciclovir or I.S.

3.3. Extraction efficiency

The recoveries of ganciclovir and acyclovir (I.S.) from spiked plasma samples were calculated by comparing the peak area of plasma spiked with standards at low, medium and high concentration levels and submitted to the sample preparation procedure, with those obtained from the analysis of corresponding directly injected standards ($n=3$). The extraction recoveries of ganciclovir and acyclovir were 95±2.36% and 105±5.36%, respectively.

3.4. Selectivity of the assay

The selectivity of the assay was determined by analysis of blank plasma from different subjects, with and without internal standard. As Fig. 2 shows, there are no interfering peaks present at the retention time for ganciclovir peaks. Sometimes a small peak waas detected near the area where acyclovir eluted. However, acyclovir peak was resolved sufficiently from this peak in the extracts. There was no interference of ganciclovir and acyclovir with other drugs, such as azathiopirine, prednisone, omeprazole, lorazepam, ranitidine, ofloxacin, acetyl-salicilic acid, paracetamol, aztreonam, vancomycin, nystatin, amiodarone, diazepam, ornidazole, cefonicid, ciclosporine, nifedipine, caffeine, allopurinol and theofilline. This suggests a lack of interference from endogenous and exogenous compounds in human plasma.

3.5. Sensitivity of the assay

The detection limit of the assay method was determined by repeated analysis of blank samples. The estimated limit of detection for ganciclovir was 10 ng/ml in plasma. The estimated limit of quantification (LOQ) was 50 ng/ml. This LOQ was confirmed in plasma using calibrators with nominal concentration 50 ng/ml. The result was 52.4±1.15 ng/ml ($n=5$) with a coefficient of variation <15%.

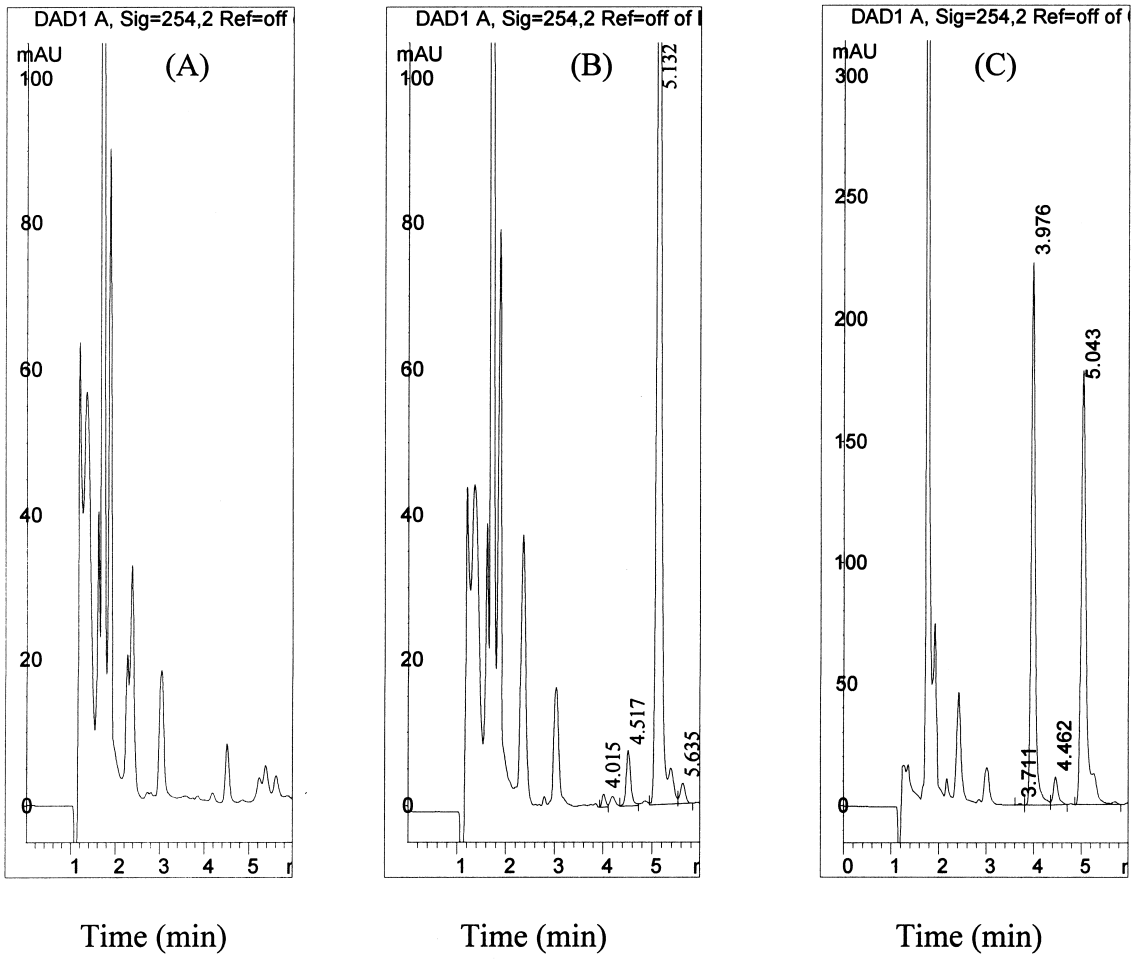

Fig. 2. Chromatograms resulting from the analysis of blank human plasma (A), human plasma spiked with 50 ng/ml and I.S. (B), and the plasma sample (6.7 μg/ml) obtained at 2 min post-infusion from a subject who received a single 5 mg/kg intravenous infusion dose of ganciclovir (C), respectively. Retention times: ganciclovir=3.97–4.0 min, Acyclovir (I.S.)=5.0–5.1 min.

3.6. Linearity of the assay

Calibration curves were obtained by plotting the ratio between the peak area of ganciclovir to the peak area of acyclovir (I.S.), against the corresponding concentrations of ganciclovir in spiked plasma. Linear least-square regression analysis of the calibration graph in three different days demonstrated linearity between the response and the corresponding concentration of ganciclovir over the range 50–10 000 ng/ml. Table 1 shows the equations of the standard curves of ganciclovir in plasma in three different days. The results of linear regression analysis show that the correlation coefficients of all standard curves are ≥0.999.

In addition, the accuracy of the assay for all the concentration values of the standard curves have been validated (Table 2).

Table 1
Standard curves for HPLC assay of ganciclovir in plasma

	n	Slope	Intercept	*r*
Day 1	6	0.0001743	0.00187	0.9990
Day 2	6	0.0001754	0.00016	0.9998
Day 3	6	0.0001713	−0.001437	0.9994

Table 2
Statistical evaluation of the analysis results for ganciclovir in standard curves along three days

Concentration added (ng/ml)	Concentration found (ng/ml)	
	Concentration found (mean±S.D. $n=3$) (ng/ml)	Relative error (%)
50	49.97±6.45	−0.07
100	98.06±4.36	−1.93
500	484.86±10.05	−3.03
1000	1018.57±34.55	1.86
5000	4999.22±99.78	−0.02
10 000	9999.31±45.89	−0.01

3.7. Accuracy of the assay

Computing the relative error of the mean analytical data from the spiked concentration assessed the accuracy of the assay method. The acceptance criterion was set at 15%.

The accuracy values in intra-day variation studies at low, medium and high concentrations of ganciclovir in plasma were within acceptable limits ($n=5$) (Table 3).

3.8. Precision of the methods

The precision of the method was expressed by the coefficient of variation (C.V.) of intra- and inter-day variations of the assay. The acceptance criterion was set at 15%.

3.8.1. Intra-day variability of the assay

The intra-day variability of the assay method was determined by the repeated analysis of quality control samples at low, medium and high concentrations ($n=5$) on the same day. The results are shown in Table 4. These data indicate that the assay method is reproducible within the same day.

3.8.2. Inter-day variability of the assay

The inter-day variability of the assay method was determined by the repeated analysis of quality control samples at low, medium and high concentrations ($n=3$) on three different days. The quality control samples were prepared as a single batch on the same day at each concentration, and then divided in aliquots that were stored at −20°C until analysis. The results are shown in Table 4. These data indicate that the assay method is reproducible on different days.

3.9. Stability of ganciclovir

No changes in ganciclovir concentrations were detected in working standard solutions after one month of storage at 4°C. The standards in plasma were stable for at least 5 h at room temperature. Ganciclovir is stable in biological matrices when stored at −20°C for at least six months [10].

Table 3
Accuracy of the HPLC method for determining ganciclovir concentrations in plasma samples

Concentration added (ng/ml)	Concentration found (ng/ml)	
	Concentration found (mean±S.D. $n=5$) (ng/ml)	Relative error (%)
100	104.87±6.13	4.87
1000	1060.64±31.12	6.06
10000	10500.67±261.749	5.01

Table 4
Inter- and intra-day precision of the HPLC method for determining ganciclovir concentrations in plasma samples

Concentration added (ng/ml)	Intra-day precision ($n=5$)		Inter-day precision (n=5)	
	Concentration found (mean±S.D.) (ng/ml)	C.V. (%)	Concentration found (mean±S.D.) (ng/ml)	C.V. (%)
100	104.870±6.13	5.85	99.470±6.62	6.65
1000	1060.639±31.12	2.93	1020.053±46.93	4.60
10000	10500.666±261.79	2.49	10033.879±209.96	2.09

4. Discussion

Ganciclovir is an antiviral agent chemically analogous to nucleosides. The bioanalysis of antiviral agents is complicated because they tend to be structurally similar to endogenous substances, requiring highly selective bioanalytical methodology. The need for analytical selectivity is particularly critical for nucleoside analogues, such as ganciclovir. Nucleoside analogues tend to be extensively metabolized to products that can coelute with ganciclovir. Therefore, chromatographic methods for the analysis of antiviral drugs in biological fluids must be capable of separating and quantitating the metabolites as well as parent compounds [7].

Antiviral drugs also exhibit substantial intra- and inter-subject variability in their absorption, distribution, metabolism and elimination leading to wide variability in plasma and tissue concentrations. Because of variability, the range of concentrations that can be analyzed for this method may be longer.

The investigations into the pharmacokinetic profile of all compounds needs an assay method that must be simple, rapid, precise, sensitive and capable of being applied to analyze plasma and tissues in the presence of other drugs. The presence of an internal standard in an assay method allows compensation for procedural losses, which might be incurred during extraction, dilution, filtration or chromatographic manipulations. The internal standard might be chemically similar to drug that is assayed.

The internal standard utilized in other methods is acyclovir, a chemical analogue of ganciclovir. This molecule is a good choice because of its solubility in mobile phase and its chromatographic and light-absorption properties, which are similar to ganciclovir.

Ganciclovir contains acidic and basic functional groups. pK_a values associated with the dissociation of a hydroxyl group and with the protonation of nitrogen in guanine, were 9.4 and 2.2. At pH 6.6 ganciclovir is a neutral form, so it can be analyzed by reversed-phase HPLC. The use of triethylamine as a component of the mobile phase enhances chromatographic peak shape for ganciclovir and acyclovir. Triethylamine, an organic modifier competes with free silanol groups of the stationary phase's column, inactivating them and therefore, avoiding the development of tailing peaks.

Ganciclovir and acyclovir show relatively short retention times in our technique. However the running time of the chromatogram has to be increased because of a severe distorted band eluted at 7 min. So the duration of the chromatogram is 15 min. This time allows the analysis of a large number of samples still in a short range of time. The absence of interfering chromatographic bands eluting in the region of ganciclovir, and the high extraction efficiencies of the extraction procedure allowed the lower reproducible limit of the assay to be 50 ng/ml. Lower quantification limits can be achieved in plasma by increasing the injection volume, or by increasing the volume of the sample.

The extraction procedure eliminates the endogenous interferences, which are frequently present in trichloroacetic and perchloric extracts of plasma.

The HPLC assay method presented here is rapid, sensitive, specific and robust. This assay method has demonstrated significant improvement in the extraction efficiency, sensitivity and specificity in analyzing ganciclovir in plasma over other existing assay methods. This assay is suitable for routine analysis of clinical plasma samples and for determination of the pharmacokinetics of ganciclovir (see Fig. 3). This

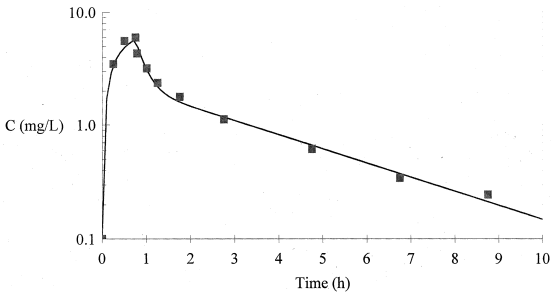

Fig. 3. Example of an application of the method: time–concentration profile of ganciclovir in plasma after an intavenous infusion dose of 5 mg/kg during 45 min.

method might be employed, for its high sensitivity, in the analysis of ganciclovir concentrations in other biological tissues.

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