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Short communication

Simple reversed-phase high-performance liquid chromatography quantitation of ganciclovir in human serum and urine

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Abstract

A fast, simple, and cost-effective HPLC method for the quantitation of the antiviral drug ganciclovir is described. The serum samples are extracted with perchloric acid and neutralized with potassium phosphate buffer, and urine samples are diluted with distilled water. A reversed-phase column with isocratic elution by 15 m*M* potassium phosphate buffer (pH 2.5) containing 0.25% acetonitrile is used to separate ganciclovir; quantitation is by UV absorbance at 254 nm. Total turnaround time is 22 min; more than 3000 samples can be run on a single column without loss of peak quality. The limit of quantitation is 0.05 μg/ml. Recoveries varied from 91 to 107% with coefficients of variation ranging from 0.387 to 7.95%.

Keywords: Ganciclovir

1. Introduction

The antiviral drug ganciclovir (9[(1,3-dihydroxy-2-propoxy)methyl]guanine; DHPG), a synthetic deoxyguanosine analog, has been shown by a number of clinical studies to be effective against cytomegalovirus in immunocomprimised patients [1–4]. In AIDS patients, ganciclovir prolongs time to progression of cytomegalovirus retinitis; effectiveness against AIDS-related gastrointestinal and pulmonary infection has also been demonstrated. In addition, ganciclovir has been shown to prevent cytomegalovirus infection in immunocomprimised organ transplant patients.

A number of pharmacokinetic studies have been done to determine absorption, distribution, and optimal dosing of ganciclovir [1,5–7]. In these studies, ganciclovir concentrations were determined by reversed-phase high-performance liquid chromatography. Though adequate for the task, these procedures have long turnaround times and marginal reproducibility at concentrations below 0.5 μg/ml. The most serious shortcoming, in our hands, was limited column life; even with ultrafiltration of the extracted sample and regular changes of guard column cartridges, columns rarely lasted more than one hundred assays before a decrease in peak quality was noted. For large-scale pharmacokinetic studies, a procedure with high sensitivity and reproducibility, short turnaround time, and extended column life was required. The procedure described here involves isocratic elution of drug from a reversed-phase column, shows superior reproducibility at 0.05 μg/ml, has a turnaround time of 22 min, and can be run

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more than 3000 times on the same column without loss of peak quality.

2. Experimental

2.1. Materials

Reagent-grade perchloric acid, phosphoric acid, potassium phosphate, trichloroacetic acid, and potassium hydroxide, and HPLC-grade water and acetonitrile were obtained from Fisher (Fair Lawn, NJ, USA). Ganciclovir was obtained from Syntex (Palo Alto, CA, USA). Standard serum was obtained from ICN (Costa Mesa, CA, USA). Ganciclovir calibration standards at concentrations 0.05, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16 μg/ml were made by serial dilution in control serum of a 320 μg/ml aqueous standard; concentrations were checked by ultraviolet absorbance at 251 nm (ε=11 800 mol^{-1} cm^{-1} in water). Serum standards were stored frozen in aliquots. Disposible sample prefilters (0.45 μm) were purchased from MSI (Westboro, MA, USA). For the analysis of ganciclovir in urine, calibration standards were prepared by dissolving solid ganciclovir in distilled water–normal urine (9:1) at concentrations 2.5, 5, 10, 20, 40, 80, 160, 320, and 640 μg/ml, and then stored frozen in aliquots.

2.2. Sample preparation

Patient samples were stored at −20°C prior to analysis. Serum samples were thawed, and 500 μl of serum was mixed with 500 μl of 0.8 *M* perchloric acid. Samples were incubated for 10 min on ice, and were then centrifuged at 12 000 *g* for 3 min to remove the precipitated protein. The samples were neutralized by mixing 660 μl of supernatant with 100 μl of 2 *M* potassium phosphate buffer (pH 8.0). The samples were then placed on ice for 10 min, and the precipitated potassium perchlorate was removed by centrifugation at 12 000 *g* for 3 min. The supernatant (pH 6.5–6.8) was stored for no more than 48 h at 4°C prior to analysis.

Alternative methods of deproteinization included ultrafiltration of the sample on a Centrifree (30 000 M_r cut-off) disposible filter (Amicon, Beverly, MA, USA) or precipitation of the protein with trichloroacetic acid. For the latter procedure, 500 μl of serum sample was mixed with 500 μl of 20% (w/v) trichloroacetic acid in water. The precipitated protein was removed by centrifugation for 3 min at 12 000 *g* and the supernantant (approximately pH 2.6) was used for analysis. In each case, residual protein concentration was determined by the method of Lowry [8].

The effect of various sample handling conditions was investigated by subjecting standards to the following conditions. The effect of heat treatment (to inactivate HIV) on ganciclovir quantitation was investigated by heating some samples at 58°C for 70 min. Other sample handling conditions which were investigated included allowing serum samples to sit at room temperature for 5 days prior to processing, storing processed samples on-instrument (at room temperature) for 48 h, and subjecting samples to 4 cycles of freeze–thaw. The effect of analyzing heparin-plasma rather than serum was investigated by analyzing plasma and serum samples drawn simultaneously from the same subject.

For the analysis of ganciclovir in urine, 0.5 ml of urine was diluted with 4.5 ml of distilled water. These diluted samples were stored for no more than 48 h at 4°C prior to analysis.

2.3. Apparatus

The HPLC system consisted of two Beckman 110B pumps, a Beckman 421A controller (Beckman Instruments, Fullerton, CA, USA), a CR1A integrator (Shimadzu, Columbia, MD, USA), a Spetraflow 757 ultraviolet detector (Kratos, Foster City, CA, USA), and a SP 8875 autosampler (Spectra Physics, San Jose, CA, USA). The column used for the majority of these studies was a Beckman Ultrasphere C_{18} 250×4.6 mm (5 μm particle size); virtually identical results were obtained from Adsorbosphere (Alltech, Deerfield, IL, USA), and Jones Chromatography (Hengoed, UK) columns of the same specifications. A 10×4.6 mm guard column cartridge of the same material was used with each column.

2.4. Chromatographic procedure

Two buffers were used for the analysis of ganciclovir. Buffer A consisted of 15 m*M* potassium

phosphate pH 2.1–3.4 with 0.25% acetonitrile. Buffer B consisted of 80% acetonitrile in water. The buffers were filtered through a 0.45-μm filter and degassed under vacuum before use. The sample (100 μl of serum sample or 10 μl of urine sample) was injected by autoinjector and eluted isocratically with buffer A for 12 min; the column was then flushed with buffer B for 5 min and reequilibrated with buffer A for 5 min. The flow-rate was constant at 1.75 ml/min. Ganciclovir eluted as a sharp peak at 9.05 min. Quantitation of ganciclovir was by ultraviolet absorbance at 254 nm. Ten-point calibration curves were prepared for serum or urine samples using the previously specified concentrations. Unweighted linear least-squares regression was performed on the peak area vs. concentration data from the calibration standards to generate a calibration curve of the form concentration=m(peak area)+b. To ensure reproducibility, a quality control sample consisting of 0.15, 1, or 5 μg/ml ganciclovir (for serum samples) or 25, 100, or 400 μg/ml ganciclovir (for urine samples), which was prepared and stored in the same manner as the calibration standards, was analyzed after every 8 patient samples.

2.5. Precision and accuracy

To test precision and accuracy within runs, four aliquots of each of the quality control standards were analyzed four times each on four different days. To test precision and accuracy between runs, each of the QC standards was analyzed on four different days. To determine within-run and between-run precision and accuracy at the limit of quantitation (serum) 0.050 μg/ml standards were analyzed in quintuplicate on two different days.

3. Results and discussion

3.1. Measurement of ganciclovir

Chromatograms of ganciclovir standards in serum extracted by the perchloric acid method are shown in Fig. 1. Of several pH values tried, optimal results were obtained in the range pH 2.5–2.9; slightly higher or lower pH values did not adequately separate ganciclovir from all serum components. In addition, use of a pH above 2.9 significantly shortened column life; degradation of peak quality was noted after approximately 100 analyses, whereas more than 3000 analyses could be performed at pH 2.5 with no loss of quality.

All three extraction methods were comparable in their ability to remove protein. All three showed between 99.5 and 99.8% protein removal. Peak shape was slightly better with the perchloric acid extraction procedure than with the trichloroacetic acid procedure; this may have been due to the lower pH or higher ionic strength of the trichloroacetic acid-extracted sample. All patient samples were extracted with perchloric acid as outlined above.

No difference in ganciclovir quantitation was noted between plasma and serum (Table 1). In each case, variation was <2%. Similarly, no differences in quantitation were produced by heat treatment, storage of serum samples for 5 days prior to processing, storage of prepared samples for 2 days on instrument at room temperature, or four cycles of freeze–thaw; in each case, the variation was <3% (data not shown).

3.2. Precision and accuracy

Standard curves consistently gave R^2 values above 0.999. Back-calculation of standards gave variations of <10% from the nominal concentrations in every case (data not shown). Typically, a new standard curve was analyzed and calculated every 100–300 samples. Within-run and between-run data for quality control samples (serum) are shown in Table 2. Mean recoveries ranged from 91 to 107%, for the quality control samples, and from 81 to 115% at the limit of quantitation. Within-run coefficients of variation ranged from 0.76 to 7.9% for the quality control samples; the coefficient of variation at the limit of quantitation was 10.2%. Between-run coefficients of variation ranged from 0.39% to 2.84% for the quality control samples; at the limit of quantitation the within-run coefficient of variation was larger than the between-run value. Similar results were obtained with urine (data not shown). Mean recoveries ranged from 98.7 to 103% within-run and 100 to 102% run-to-run. Within-run coefficients of variation ranged from 0.63 to 1.2%. Between-run coefficients of variation ranged from 1.1% to 3.2%.

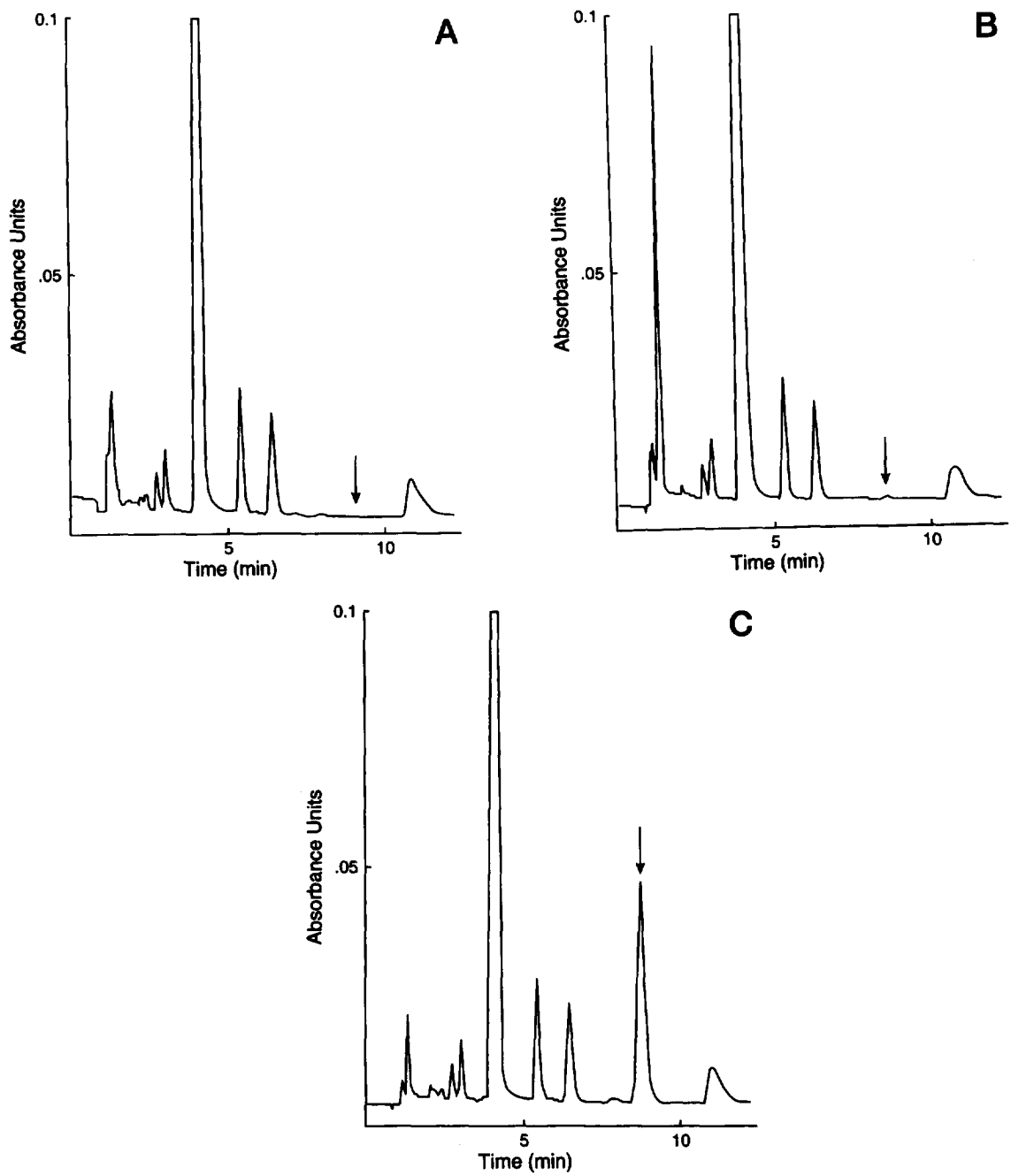

Fig. 1. Chromatograms of serum blank (A) and ganciclovir at 0.050 μg/ml (B) and 4 μg/ml (C). Ganciclovir peak is indicated by the arrow at 9.05 min.

When samples were extracted with the perchloric acid method and analyzed at pH 2.9, loss of peak quality was noted after approximately 2000 analyses; if the extracted sample was also filtered with a disposible prefilter, more than 3000 analyses could be run with no loss of peak quality. Guard column

Table 1
Comparison of ganciclovir quantitation in serum and plasma

Subject	Ganciclovir concentration (μg/ml)		
	Serum	Plasma	Deviation (%)
1	3.087	3.098	0.36
1	2.833	2.815	0.64
2	1.042	1.036	−0.5
2	0.826	0.835	1.09
3	2.653	2.680	1.02
4	2.746	2.765	0.69
4	3.370	3.367	−0.09

Table 2
Precision and accuracy of serum ganciclovir quantitation

	Ganciclovir concentration (μg/ml)			
	0.050	0.20	1.00	5.00
Repeats per day	5	4	4	4
Number of days	2	4	4	4
Grand mean	0.0513	0.196	0.999	5.020
Within-run C.V. (%)	10.2	7.95	1.21	0.757
Between-run C.V. (%)	–[a]	2.84	1.50	0.387
Recoveries (%)	81–115	91–107	97–102	99–102

[a] Between-run C.V. (%)≪within-run C.V. (%).

cartridges were typically replaced every 500 analyses.

The above method has been used for more than 10 000 analyses in our laboratory. The presence of interfering peaks that caused an analysis to be rejected was less than 0.5%. When used with automated equipment, more than 400 analyses per week can be performed. Through long-term use this method has proven to be a simple, inexpensive, and highly reliable method for the analysis of ganciclovir in clinical samples.

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References

[1] A. Markham and D. Faulds, Drugs, 48 (1994) 455.
[2] A.E. Duke, D.F. Smee, M. Chernow, R. Boehme and T.R. Matthews, Antiviral Res., 6 (1986) 299.
[3] S. Shigeta, K. Konno, M. Baba, T. Yokota and E. DeClercq, J. Infect. Dis., 163 (1991) 270.
[4] R. Snoeck, D. Schols, G. Andre, J. Neyts and E. DeClercq, Antiviral Res., 16 (1991) 1.
[5] M.A. Jacobson, P DeMiranda, D.M. Cederberg, T. Burnette, E. Cobb, H.R. Brodie and J. Mills, Antimicrob. Agents Chemother., 31 (1987) 1251.
[6] C. Fletcher, R. Sawchuk, B. Chinnock, P. DeMiranda and H.H. Balfour, Clin. Pharmacol. Ther., 40 (1986) 281.
[7] J.M. Trang, L. Kidd, W. Gruber, G. Storch, G. Demmler, R. Jacobs, W. Danber, S. Starr, R. Pass, S. Stagno, C. Alford, S.J. Soong, R.J. Whitley and J.P. Sommadossi, Clin. Pharm. Ther., 53 (1993) 15.
[8] O.H. Lowry, M.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.