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Short Communication

HPLC determination of serum ganciclovir using ultrafiltration, ultraviolet and fluorescence detection

MARLIES KOEL and PETER NEBINGER*

Medizinisches Labor Dr. Enzenauer & Dr. Wilhelm, Postfach 3909, D-4500 Osnabrück, Germany

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Introduction

Ganciclovir or 9-(1,3-dihydroxy-2-propoxymethyl)guanine represents an acyclic deoxyguanosine analogue and is a potent inhibitor of viral replication of the herpes family, including Epstein–Barr and cytomegalovirus [1, 2]. Individuals with suppressed cellular immunity are in danger of these infections. The main side effect associated with ganciclovir therapy is the suppression of the bone marrow function [3, 4]. Furthermore, these patients are on a multiple drug therapy and drug–drug interactions or reduced renal function may occur. These factors demonstrate the importance of monitoring the concentrations in the serum during ganciclovir therapy.

Several methods for ganciclovir determination including HPLC [5–7], radioimmunoassay [8], and enzyme-linked immunosorbent assay [9] have been reported. The immunological methods are more sensitive than the HPLC methods with a reported detection limit of 0.1 μg l^{-1} but they require lengthy procedures for the assay performance. In this paper a very simple, rapid and sensitive HPLC method using heat inactivation, to avoid infectional risks, and ultrafiltration as sample pre-treatment is described.

Experimental

Materials

Ganciclovir was a generous gift from the Syntex Company (Aachen, Germany). The internal standard guanosine was purchased from Aldrich Chemicals (Steinheim, Germany), the ultrafiltration units Centrisart I (2.5 ml, 5000 dalton) from Sartorius (Göttingen, Germany) and 1-octanesulphonic acid from Fluka Chemicals (Neu-Ulm, Germany). All other reagents used were of the highest quality available.

Standard solutions

Stock 1 mg ml^{-1} solutions of ganciclovir and the internal standard guanosine were prepared in methanol and stored at 4°C. These standard solutions are stable for at least 1 month. Standard curves were prepared by adding aliquots of ganciclovir to human drug-free serum to give final concentrations of 0.025, 0.05, 0.1, 0.5, 1, 2, 5, 7.5, 10, 12.5, 15, 17.5, 20, 50, 75 and 100 mg l^{-1}.

Sample preparation

Prior to the study, serum samples were treated at 56°C for 1 h to inactivate viruses. A 500 μl volume of heat inactivated serum specimen and 50 μl of the internal standard (100 mg l^{-1} for UV-detection, 200 mg l^{-1} for fluorescence detection) were mixed prior to ultrafiltration. The ultrafiltration step was processed according to the manufacturer's recommendations using Centrisart I ultrafiltration units. The obtained ultrafiltrate was diluted 1:30 for ultraviolet and 1:60 for fluorescence detection with eluent buffer. A 50 μl volume was injected for the HPLC analysis.

* Author to whom correspondence should be addressed.

Instrumentation and chromatographic conditions

For the HPLC analysis a HPLC system consisting of a L-6000 HPLC pump, a L-4250 UV–vis detector, a F 1050 fluorescence detector and a D-2500 integrator (Merck, Darmstadt, Germany) were used. A Lichrospher RP8e (125 mm × 4 mm i.d., 5 μm, endcapped) column was employed. The mobile phase consisted of 5% methanol and 95% 0.05 mmol l^{-1} 1-octanesulphonic acid in 0.1 mol l^{-1} phosphate buffer pH 3.0 (v/v). The flow rate was 1.0 ml min^{-1}. The column temperature was ambient and the effluent was monitored at 254 nm and by fluorescence with excitation wavelength at 285 nm and emission wavelength at 380 nm.

Results and Discussion

Sample preparation and chromatography

Ganciclovir reveals a high water solubility

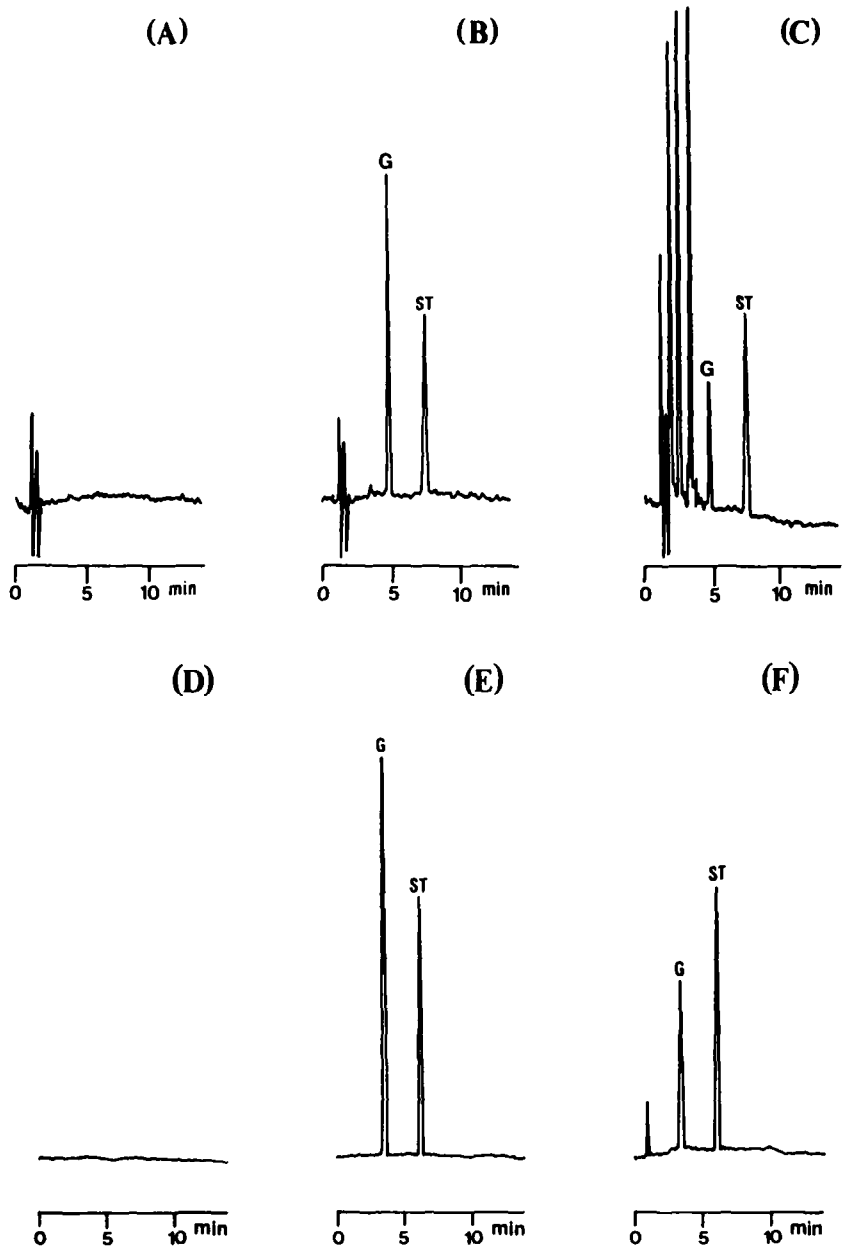

Figure 1
Typical chromatograms for the determination of ganciclovir in human serum monitored by ultraviolet (A)–(C) and fluorescence detection (D)–(F). (A), (D) Blank human serum; (B), (E) blank human serum spiked with 10 mg l^{-1} ganciclovir and internal standard; (C), (F) patient serum sample with 3.8 mg l^{-1} ganciclovir. G = Ganciclovir; ST = internal standard (guanosine).

Table 1
Precision data* of ganciclovir determination using ultraviolet (A) and fluorescence detection (B)

		Precision at ganciclovir concentrations (mg l^{-1}) of:			
		0.1	5.0	15.0	30.0
A	Intra-run	0.08 (6.1)	5.02 (4.1)	14.92 (2.8)	29.83 (1.9)
	Inter-run	0.07 (7.5)	4.96 (4.9)	14.79 (3.7)	29.79 (2.9)
B	Intra-run	0.09 (4.0)	4.99 (2.7)	14.97 (2.0)	29.98 (1.4)
	Inter-run	0.08 (5.3)	4.98 (3.0)	14.95 (2.4)	29.93 (1.8)

*Data are presented as means, with the relative standard deviations in parentheses.

and a low protein binding of 1–2% [5]. Recently published sample purification methods include time consuming two-step extraction procedures [5] or protein precipitation methods which are disadvantageous because of serum impurity problems in chromatography [6]. For this reason ultrafiltration was chosen as a simple pre-treatment technique taking advantage of the low protein binding of ganciclovir [10].

Typical chromatograms obtained by analysing blank human serum and serum spiked with ganciclovir are given in Fig. 1(A), (B), (D), (E). The blank serum is shown to be free of endogenous interferences. The retention times of ganciclovir and guanosine are 4.71 and 7.35 min, respectively. A typical analysis of a patient sample is given in Fig. 1(C), (F). In all analysed patient samples no interferences were observed by endogenous compounds or other medicaments like acyclovir, zidovudine. The superiority of fluorescence detection is demonstrated in Fig. 1(F). In comparison to the ultraviolet detection less impurity problems arise and fluorescence detection is more specific and sensitive.

Analytical validation

The mean recovery of ganciclovir is in the range of 98–100% for the tested concentrations of 0.1–100 mg l^{-1}. The detection limit is 0.05 mg l^{-1} for ultraviolet detection and 0.01 mg l^{-1} for fluorescent detection with a signal-to-noise ratio of 5:1. The detection limit can be further increased by using a lower dilution factor of the obtained ultrafiltrate. Ganciclovir shows an excellent temperature stability in serum. Repeated freezing at −20°C, storage at room temperature for 1 week and heat inactivation at 56°C for 1 h showed no decomposition or significant loss in concentration.

Linearity and precision

A linear calibration curve for height versus ganciclovir concentration was obtained in the range of 0.05–100 mg l^{-1} (ultraviolet detection) and 0.01–50 mg l^{-1} (fluorescence detection). This calibration curve fully covers the therapeutic range of ganciclovir which is given with 0.1–15 mg l^{-1}. To determine intra-run and inter-run precision, each of the reference samples were tested 10 times in a single run and 10 times in separate runs (Table 1). For ultraviolet detection relative standard deviation were in the range of 1.9–7.5%. Fluorescence detection showed lower values of 1.4–5.3%. The mean calibration curves (n = 5) were $y = 0.160x + 0.04$ ($r > 0.998$) for ultraviolet detection and $y = 0.187x + 0.01$ ($r > 0.999$) for fluorescence detection, where y = peak height ratio and x = sample concentration.

In conclusion, the described simple and specific HPLC fluorescence method shows in comparison to the ultraviolet HPLC method the best relative standard deviations, linearity ($r > 0.999$), excellent sensitivity and reliability for the measurement of ganciclovir in a wide variety of clinical situations. This method can also be used for pharmacokinetic and metabolism studies.

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