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Research Article

Quantification of ganciclovir in human plasma using capillary electrophoresis

A fast, simple, specific capillary electrophoretic method in the MEKC mode for the quantification of the antiviral drug ganciclovir is described. The separation was obtained using a 50 μm id fused-silica capillary, 60 mM borax buffer (pH 9.25) containing 40 mM SDS using ethenoadenosine as the internal standard. Sample preparation was done by ultrafiltration with a Microcon 30 000 kDa filter. The analytes were detected with UV detector at 254 nm. A sufficient sensitivity was achieved by using a bubble cell capillary. The linear range was from 0.5 to 10 mg/L with a LOQ of 0.5 mg/L. Correlation coefficients were better than 0.999 whereas inter- and intraday precision and accuracy were less than 10.7%. The analysis of patients' samples after administration of ganciclovir indicates that the method is suitable for drug monitoring in the clinic.

Keywords: Acyclovir / Bubble cell / Ganciclovir / Plasma / Ultrafiltration
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1 Introduction

Ganciclovir, 9-(1.3-dihydroxy-2-propoxymethyl) guanine **(1)**, a guanine derivative nucleoside analogue, is an antiviral drug showing activity against cytomegalovirus (CMV), variccella-zoster virus, herpes virus and Epstein-Barr virus [1, 2]. Ganciclovir is mainly used in the treatment of CMV infection in immunocompromised patients after bone marrow or organ transplantion. After phosphorylation by viral kinase to ganciclovir triphosphate it displays affinity for DNA polymerase and is a competitive inhibitor of deoxyguanosine triphosphate resulting in inhibition of DNA replication [3]. Ganciclovir is the first choice in immunocompromised patients. The treatment with ganciclovir has been associated with serious toxic side effects such as neutropenia, thrombocytopenia and anaemia. Monitoring of ganciclovir plasma concentrations is applied in the case of therapeutic failure to check if sufficient plasma levels are achieved [4]. In addition, therapeutic drug monitoring is important in patients who have acute or chronic renal impairment and in children. The elimination of ganciclovir is mainly renal.

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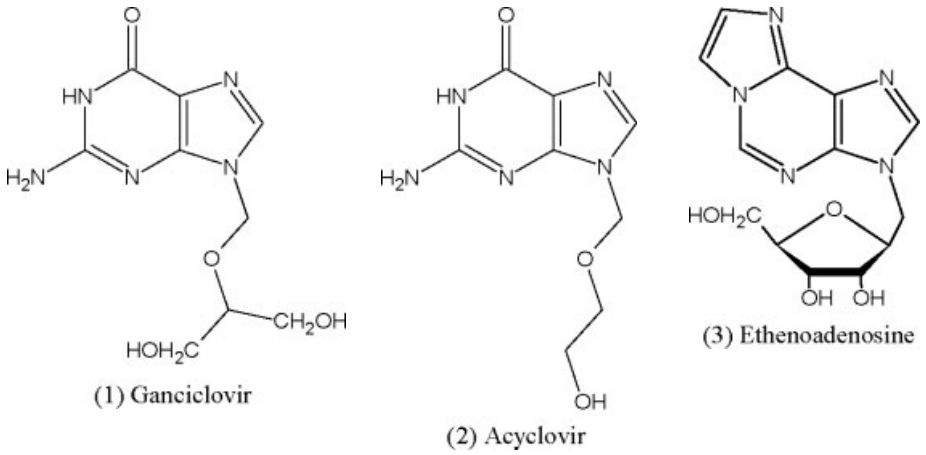
Abbreviations: CMV, cytomegalo virus, **IS**, internal standard

Ganciclovir is structurally similar to endogenous purines making the analysis complicated and requires the use of highly selective analytical methods. The analysis of ganciclovir in plasma has been described by several HPLC methods employing different modes of detection such as UV, fluorescence, MS and pulsed amperometric detection [5–10]. The use of CE for the determination of drug levels in pharmacokinetic studies and drug monitoring is an alternative to HPLC with its high separation efficiency, ease of operation, speed and small sample volume requirement [11, 12]. These advantages make it a suitable tool in the bioanalysis of plasma concentrations especially in children. CE methods for the quantification of acyclovir **(2)** have been described recently [13, 14]. However, these methods were not appropriate for ganciclovir analysis. Our aim was to develop a simpler and fast method for the determination of ganciclovir suited for routine application.

2 Materials and methods

2.1 Materials and apparatus

The separation was performed on a Beckman P/ACE 5510 system equipped with a UV detector. Fused-silica capillaries of 40 cm effective length and an id of 50 μm (od 375 μm; Beckman Instruments, München, Germany) and bubble cell capillaries (50 μm, od 375 μm) from Agilent Technologies (Waldbronn, Germany) were used.



Scheme 1. Structures of the analytes

Ganciclovir was kindly supplied from Roche (Basel, Switzerland), the internal standard (IS) ethenoadenosine **(3)** and acyclovir 99% powder were from Sigma-Aldrich (Deisenhofen, Germany). The samples were ultrafiltrated with a Microcon 30 000 kDa filter from Millipore (Schwalbach, Germany). The separation buffer was prepared using borax, sodium tetroborate decahydrate ACS reagent 99.5–105.0%, and SDS 95%, all from Sigma-Aldrich. All solutions for CE were filtered through a 0.22 μM membrane filter (Millipore, Bedford, MA, USA). Water was prepared using a Milli-Q UF Plus system Millipore. Blank plasma from healthy donors was obtained from the Department of Transfusion Medicine, University of Münster, Germany. Standard chemicals were of analytical grade.

2.2 Separation procedures

New capillaries were first rinsed with 0.1 M NaOH for 20 min followed by rinsing with the separation buffer for 30 min. To achieve the reproducible migration time, the capillary was rinsed daily with NaOH for 20 min, the running buffer for 30 min and each separation was preceded by a 2 min rinse with 0.1 M NaOH, a 1 min rinse with deionized water, followed by a 3 min rinse with the separation buffer. Samples were introduced using pressure injection at 3448 Pa. All separations were carried out at 22°C using a voltage of 15 kV. Detection was monitored at 254 nm using a UV detector.

Borax buffer was used with concentrations between 40 and 120 mM and pH values between 8.9 and 9.3, as stated in the text. The pH was adjusted using 1 M NaOH or 1M HCl. Subsequently, SDS was added to the separation buffer. Before use, the buffer was passed through a 0.22 μm filter.

2.3 Sample preparation

For sample preparation several methods were tested.

2.3.1 Deproteination with TCA

Hundred microliters of plasma was spiked with 10 μL of different standard solutions to test for suitability as the 8-chlorotheophylline: 1 g/L IS, 0.1 g/L guanosinemonophosphat, 0.1 g/L 8-azaguanine, 0.1 g/L acyclovir, 0.1 g/L adenosine, 0.1 g/L 2-chloroadenosine and 10 mg/L ethenoadenosine dissolved in water. The mixture was deproteinized with 10 μL of 50% TCA. After shaking for 30 s the samples were centrifuged at 2000 × *g* for 10 min and the supernatant was removed and neutralized with 6 μL of 2 M sodium hydroxide and subsequently extracted with 2 mL of chloroform and centrifuged at 3000 × *g*. The aqueous phase was injected to the CE system.

2.3.2 Deproteination with perchloric acid

Hundred microliters of plasma was mixed with 10 μL of perchloric acid 35% and 10 μL acyclovir 0.1 g/L dissolved in water (containing 40 mM SDS) as IS and was centrifuged at 2800 × *g* for 5 min to remove the precipitated protein. The supernatant was injected into the CE system.

2.3.3 Ultrafiltration

Hundred microliters of the sample mixed with 10 μL acyclovir 0.1 g/L dissolved in water containing 40 mM SDS, 2-chloradenosine 0.1 g/L or ethenoadenosine 10 mg/L as IS and filtered through a Microcon 30 000 kDa filter at 2800 × *g* for 5 min.

3 Results and discussion

The method development was based on the previously described CE methods for acyclovir [13, 14]. The quantification of plasma samples down to a concentration of 0.5 mg/L is necessary for the clinical application of the method. It was obvious from the structural properties that a basic buffer is required for the assay of ganciclovir. Addition of SDS to the running buffer was necessary to achieve baseline separation of the compounds of interest from plasma constituents. A lower concentration of SDS in the sample matrix, added with the IS solution, provides a better separation due to a stacking effect [15]. While separation of ganciclovir and related compounds in aqueous solutions was easy to achieve, different sample preparation procedures had to be tested to get a reproducible separation in human plasma as numerous compounds with similar structures are present.

3.1 Sample preparation with trichloroacetic acid

The operating parameters were as follows: pressure injection with 3448 Pa for 30 s, and a running buffer of 90 mM borax containing 40 mM SDS (pH 9.2). Under these conditions, ganciclovir could be detected easily; however, it was difficult to find a suitable IS 8-Chlorotheophylline, guanosine-monophosphate, and 8-azaguanine gave no peaks in the detection window. The signal of acyclovir overlapped with the ganciclovir peak while with adenosine, 2-chloroadenosine, and ethenoadenosine, asymmetric peaks occurred.

3.2 Sample preparation with perchloric acid

The operating parameters were as follows: pressure injection with 3448 Pa for 40 s and a running buffer of 90 mM borax containing 80 mM SDS (pH 9.2). Acyclovir dissolved in 40 mM SDS was used as IS Ganciclovir could be detected with good peak symmetry and the separation from plasma interferences was sufficient. A concentration of 0.5 mg/L could be easily detected under these conditions. However, the intraday precision had a SD of 21%, because peak splitting sometimes occurred with acyclovir. To overcome these difficulties the supernatant was neutralized by NaOH and an additional cleaning process with chloroform was added. However, robustness of the method was still not sufficient.

3.3 Ultrafiltration

The operating parameters were the following: pressure injection with 3448 Pa for 30 s, and a running buffer of 90 mM borax containing 80 mM SDS (pH 9.2). Acyclovir dissolved in 40 mM SDS was used as the IS The separation of the peak of acyclovir and ganciclovir from plasma constituents was not sufficient. Several buffers with different pH were tested. At pH values ranging from 8.9 to 9.1 there was no separation between ganciclovir and the IS while at pH values above 9.1, plasma peaks overlapped the ganciclovir peak. Therefore, a borax buffer containing 120 mM borax and 90 mM SDS (pH 9.0) was tested with the IS 2-chloradenosine. The separation due to plasma interference and between ganciclovir and IS was sufficient. The average intraday precision at concentrations of 0.5, 2 and 10 mg/L was of 7, 7, and 11%, respectively ($n = 15$). However, under these conditions the results could not be reproduced over several days and other conditions had to be tested. Therefore, borax buffer with a lower borax concentration of 60 mM and a pH of 9.25 containing 40 mM SDS was used in the following experiments. The separation of ganciclovir and the IS ethenoadenosine from plasma interference was sufficient (Fig. 1). Acyclovir could not be used as the IS because its peak overlapped the peak of ganciclovir. Also, no interfering peaks in the blank plasma were observed. A bubble cell capillary was used resulting in an increase in sensitivity of factor 2. Under these conditions, the required LOQ of 0.5 mg/L could be achieved (Fig. 2). Plasma concentrations of ganciclovir after i.v. administration of 5 mg/L every 12 h in the therapy and prophylaxis for CMV disease were 3.03 $\pm$ 2.63 mg/L [16]. After oral administration of 1000 mg every 8 h, the mean plasma concentrations were 0.54 mg/L in [17]. In general, after oral administration, the trough levels are between 0.2 and 0.5 mg/L and should be higher than 0.5 mg/L [18]. The median concentration of ganciclovir inhibiting CMV replication (IC_{50}) *in vitro* ranges from 0.26 to 1.28 mg/L [18]. Thus, the sensitivity of the CE method is sufficient to quantify the

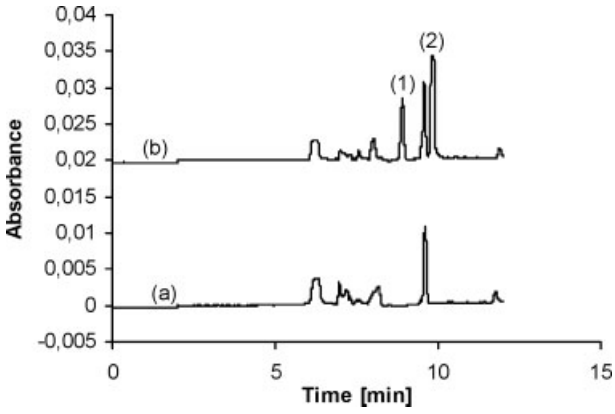


Figure 1. (a) Blank plasma. (b) Separation of ganciclovir 5 mg/L (1) and the IS (ethenoadenosine, 2) in spiked plasma after ultrafiltration. Bubble cell capillary and 60 mM borax (pH 9.25) containing 40 mM SDS, injection time 30 s.

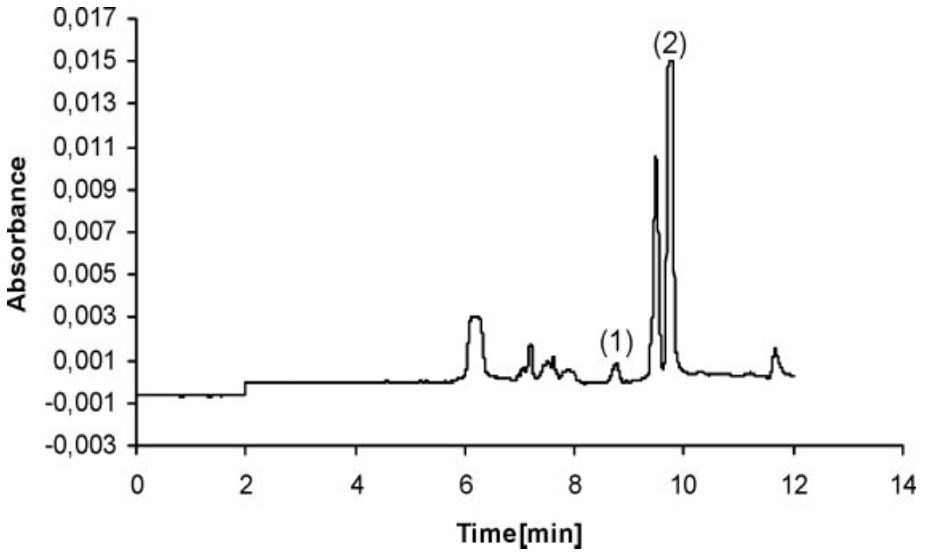


Figure 2. Separation of ganciclovir 0.5 mg/L (1) and the IS (ethenoadenosine, 2) in spiked plasma after ultrafiltration. Conditions as in Fig. 1.

plasma concentration of ganciclovir after i.v. administration. After oral administration, the sensitivity might not be sufficient in all situations. However, substances which have a similar chemical structure to ganciclovir and may be detected under the same conditions were tested such as caffeine, xanthine and allopurinol. Only caffeine appeared earlier than ganciclovir in the electropherogram whereas the other drugs were not detected with the applied conditions.

3.4 Quantification and validation

Blank plasma was spiked with 0.5, 0.83, 1.25, 2.5, 5 and 10 mg/L of ganciclovir and 10 mg/L of ethenoadenosine (IS). The samples were ultrafiltrated and analysed directly by CE. Calibration curves were obtained by plotting the corrected peak area ratios (ganciclovir/IS) *versus* ganciclovir concentrations. The peak area was corrected by using the migration time of the analyte (area/migration time). Standard curves were prepared every day and the results of the linear regression showed that the correlation coefficients of all standard curves were >0.999. Furthermore, the back-calculated values of the calibration samples were found to differ by less than 10% from the nominal values.

The precision of the method was expressed as the CV of intra- and interday variation. The intraday variability of the assay method was determined by the repeated analysis of quality control samples at low, medium and high concentrations of ganciclovir ($n = 6$) on the same day. The results are shown in Table 1. The interday variability of the assay method was determined by the repeated analysis of quality control on six different days (Table 1). These data indicate that the assay method is reproducible on different days.

Table 1. Precision and accuracy of the assay for the determination of ganciclovir concentrations

Concentration added (mg/L)	Mean concentration found (mg/L)	Accuracy (%)	Precision (%)	*n*
0.5	0.46	−8.59	5.35	6
2	2.19	9.46	4.8	6
8	8.72	9.05	5.92	6
Day-to-day reproducibility				
0.5	0.48	−3.84	10.7	6
2	1.95	−2.43	5.96	6
8	8.56	6.95	7.32	6

3.5 Clinical application

The first patient's samples were analyzed using this method. Figure 3 shows an electropherogram of a sample derived from a patient with renal insufficiency. The plasma had to be diluted 1:5 with water. This patient received 75 mg of ganciclovir as a 2-h infusion every day and the sample was drawn approximately 12 h after infusion. The ganciclovir peak could easily be identified by the migration time. Other peaks which were not present in the blank plasma occurred possibly due to the renal insufficiency of the patient. These peaks, however, could not be identified. Ganciclovir concentration was found to be 4.3 mg/L. The same sample was analyzed in another laboratory

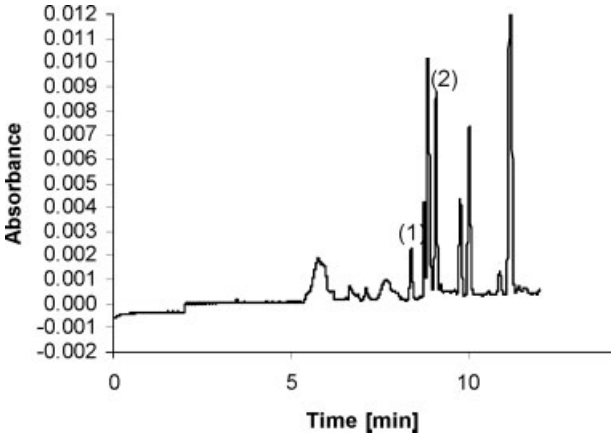


Figure 3. Plasma sample (1:5) from a patient with renal insufficiency 12 h after administration of ganciclovir. Ganciclovir (1) and IS ethenoadenosine (2). CE conditions as in Fig. 1.

using HPLC where the result was 4.15 mg/L. More plasma samples of patients will be analyzed to compare our method to the HPLC determination.

4 Concluding remarks

Ganciclovir requires therapeutic drug monitoring to achieve the optimum therapy and to minimize the adverse reactions. The CE method is more specific for the therapeutic drug monitoring or pharmacokinetic investigation than the immunoassays usually applied. This method is sensitive enough to determine the clinically relevant concentrations of ganciclovir in low plasma volumes without much organic solvent consumption. With slight modifications, it can also be applied for the quantification of acyclovir.

5 References

[1] Faulds, D., Heel, R. C., *Drugs* 1990, *39*, 597–638.
[2] Green, M., Kaufmann, M., Wilson, J., Reyes, J., *Clin. Infect Dis.* 1997, *25*, 1344–1349.
[3] Crumpacker, C. S., *N. Engl. J. Med.* 1996, *335*, 721–729.
[4] Scott, J. C., Partovi, N., Ensom, H. H., *Ther. Drug Monit.* 2004, *28*, 68–76.
[5] Campanero, M. A., Sadaba, B., Garcia-Queteglas, E., Azanza, J. R., *J. Chromatogr. B* 1998, *706*, 311–317.
[6] Page, T., Sherwood, C., Connor, J. D., Tarnowiski, T., *J. Chromatogr. B* 1996, *675*, 342–346.
[7] Kishino, S., Takekuma, Y., Sugawara, M., Shimamura, T. *et al.*, *J. Chromatogr. B* 2002, *780*, 289–294.
[8] Cociglio, M., Peyriere, H., Hillaire-Busy, D., Alric, R., *J. Chromatogr. B* 1998, *705*, 79–85.
[9] Merodio, M., Campanero, M. A., Mirshahi, T., Mirshahi, M., Irache, J. M., *J. Chromatogr. A* 2000, *870*, 159–67.
[10] Hedaya, M. A., Sawchuk, R. J., *Pharm. Res.* 1999, *7*, 1113–1118.
[11] Hempel, G., *Clin. Chem. Lab. Med.* 2003, *41*, 720–723.
[12] Thormann, W., Lurie, I. S., McCord, B., Marti, U. *et al.*, *Electrophoresis* 2001, *22*, 4216–4243.
[13] Loregian, A., Gatti, R., Palu, G., *J. Chromatogr. B* 2001, *764*, 289–311.
[14] Vo, H. C., Henning, P. A., Leung, D. T., Sacks, S. L., *J. Chromatogr. B* 2002, *772*, 291–297.
[15] Quirino, J. P., Terabe, S., *J. Chromatogr. A* 1999, *856*, 465–482.
[16] Fishman, J. A., Doran, M. T., Volpicelli, S. A., *Transplantation* 2000, *69*, 389–394.
[17] Spector, S. A., Busch, D. F., Follansbee, S., *J. Infect. Dis.* 1995, *171*, 1431–1437.
[18] McGavin, J. K., Goa, K. L., *Drugs* 2001, *61*, 1153–1183.

