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ScienceDirect
Journal of Chromatography B, 846 (2007) 281–290
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Development and validation of a capillary electrophoresis method for the characterization of herpes simplex virus type 1 (HSV-1) thymidine kinase substrates and inhibitors

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Received 20 June 2006; accepted 8 September 2006
Available online 4 October 2006

Abstract

A fast, convenient capillary electrophoresis (CE) method was developed for monitoring the enzymatic reaction of herpes simplex virus type 1 thymidine kinase (HSV-1 TK). The reaction was performed in a test tube followed by quantitative analysis of the products. The optimized CE conditions were as follows: polyacrylamide-coated capillary (20 cm effective length × 50 μm), electrokinetic injection for 30 s, 50 mM phosphate buffer at pH 6.5, constant current of −60 μA, UV detection at 210 nm, UMP or cAMP were used as internal standards. Phosphorylated products eluted within less than 7 min. The limits of detection were 0.36 μM for dTMP and 0.86 μM for GMP. The method was used to study enzyme kinetics, and to investigate alternative substrates and inhibitors.

Keywords: Capillary electrophoresis; Herpes simplex virus type 1; K_{cat} value; Michaelis-Menten analysis; Thymidine kinase assay

1. Introduction

Thymidine kinase (EC 2.7.121, TK) is the key enzyme in the pyrimidine salvage pathway catalyzing the transfer of the γ-phosphate from ATP to thymidine to produce thymidine 5′-monophosphate in the presence of Mg^{2+} (see Fig. 1) [1]. Herpes simplex virus type 1 encodes for its own TK (HSV-1 TK), which has a very broad substrate specificity; it phosphorylates a wide range of nucleosides and analogs, including acycloguanosines (e.g. aciclovir, ganciclovir, penciclovir) and uracil derivatives (e.g. 2′-fluoro-2′-deoxy-1-arabinofuranosyl-5-iodouracil (FIAU)) [2–4]. While these nucleoside analogs have been identified as substrates of the HSV-1 TK they are not recognized as substrates by human cellular kinases [5]. Antiviral nucleoside analogs, such as aciclovir, are phosphorylated in three steps in HSV-1 infected cells. The first phosphorylation is completed by the virally encoded TK, while the second and third phosphorylations to the corresponding di- and triphosphates are achieved by cellular kinases. Antiviral nucleoside triphosphate analogs may be competitive inhibitors of viral DNA polymerase, and they may act as DNA chain terminators [6]. Endogenous thymidine within the cell competes with the nucleoside analogs for the active site of the enzymes. The rate of phosphorylation by TK at a specific concentration of substrate can be determined by two ways, the K_m value and V_{max} (maximum velocity). At low concentrations (equal to K_m) of the substrate the binding affinity plays a key role, but at higher concentrations, V_{max} is the key factor in determining the phosphorylation [7].

Several methods, including radioisotopic [8–10], HPLC [11–13] and spectrophotometric [14,15] assays have been developed for quantifying the formation of phosphorylated substrates in TK-catalyzed reactions. The radioisotopic assays involve the utilization of radiolabeled substrates, such as [^{3}H]dT [3]. This method is very sensitive but time-consuming and not environment-friendly. High-performance liquid chromatography (HPLC) among the separation techniques has been used for the characterization of new antiviral lead compounds [13], but this technique suffers from the requirement of large quantities of

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doi:10.1016/j.jchromb.2006.09.018

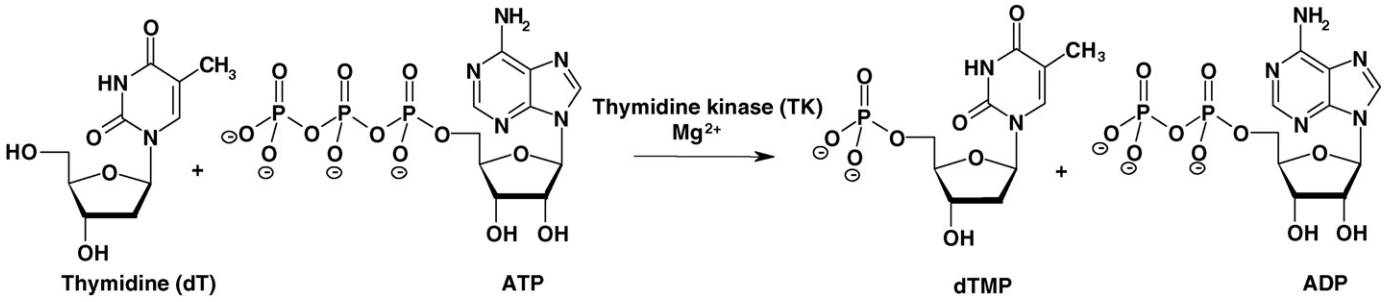


Fig. 1. Enzymatic reaction of thymidine kinase.

solvents, relatively high prices for columns, and frequently long rentention times. In addition, biological samples usually have to be pretreated to avoid clogging of the columns. Spectrophotometric methods [14] have also been employed for the determination of K_{cat} values of HSV-1 TK substrates. The described method is rather circumstantial since it makes use of two different enzymes (pyruvate kinase and lactate dehydrogenase); the sensitivity of this time-consuming procedure is limited. In addition, it requires large amounts of materials and is prone to interference from biological matrices.

Capillary electrophoresis (CE) is an excellent and easy analytical technique for enzyme assays offering the advantage of high resolution and short separation times while consuming extremely small amounts (nanoliters) of sample; radio- or otherwise labeled substrates are not required. CE systems have been successfully applied to assaying enzymatic activity [16,17], including the determination of Michaelis-Menten constants (K_m values) [18,19], inhibition constants (K_i values for enzyme inhibitors) [20,21] and catalytic constants (K_{cat}) of substrates [22]. CE is particularly useful for investigating enzymatic reactions involving charged substrates or products, e.g. for monitoring the phosphorylation or dephosphorylation reactions [23]. In addition, CE offers the possibility of automatization and can easily be performed in a high-throughput screening (HTS) assay format.

Most recently, Tzeng and Hung published a CE-based assay for the determination of thymidine-5′-monophosphate (dTMP) and thymidine-5′-diphosphate (dTDP) [24] using uncoated fused-silica capillaries. In the present study, we have developed an optimized capillary electrophoresis method with increased sensitivity using coated capillaries, that allows to measure substrates and potential inhibitors for TK within a few minutes with high accuracy and reproducibility. The new method is suitable for the high-throughput screening of test compounds.

2. Materials and methods

2.1. Materials

Adenosine-5′-triphosphate (ATP), adenosine-5′-diphosphate (ADP), thymidine (dT), uridine-5′-monophosphate (UMP), (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), thymidine-5′-monophosphate, and guanosine-5′-monophosphate (GMP) were obtained from Sigma (Steinheim, Germany). Magnesium chloride and tris(hydroxymethyl)aminomethane (Trizma Base) were from Sigma (Taufkirchen, Germany). Dipotassium hydrogen phosphate was obtained from Fluka (Neu-Ulm, Germany). Ganciclovir (GCV) was purchased from Roche (Mannheim, Germany).

2.2. Expression and purification of HSV-1 TK

HSV-1 TK was expressed as PreScission™ Protease cleavable gluthathione-*S*-transferase-fusion protein in competent *Escherichia coli* BL21 using the vector pGEX-6P-2-TK as described [12]. The protein was purified by gluthathione-affinity chromatography followed by on-column PreScission protease cleavage following the protocol described earlier [12]. Purification was monitored by SDS-polyacrylamide gel electrophoresis and led to a >90% pure HSV-1 TK. Total protein concentration was measured using the Bio-Rad protein assay.

2.3. Capillary electrophoresis apparatus and conditions

A P/ACE capillary electrophoresis system MDQ (Beckman Coulter Instruments, Fullerton, CA, USA) equipped with a UV diode-array detector (DAD) was used in this study. The CE-instrument was fully controlled through a PC, which operated with the analysis software 32 KARAT obtained from Beckman Coulter.

2.4. Method A: Fused-silica capillary with pressure injection

The electrophoretic separations were carried out using an eCAP fused-silica capillary [40 cm (30 cm effective length) × 75 μm internal diameter (i.d.) × 375 μm outer diameter (o.d.), obtained from Beckman Coulter]. The runs were performed under the following conditions: $T = 25\,^{\circ}C$, $\lambda_{max} = 260$ nm, current = 95 μA, running buffer 20 mM sodium phosphate buffer, pH 8.5. The capillary was washed with 0.1 N aqueous NaOH solution for 2 min, followed by deionized water for 1 min, and subsequently with running buffer for 1 min before each injection. Injections were made by applying 0.1 psi of pressure to the sample solution for 25 s. Rinsing of the untreated fused-silica capillary with 0.1N NaOH solution between the runs

was required in order to remove the cations from the capillary wall and to provide the same silica surface for each run [25]. At the beginning of each working day, the capillary was washed with 0.1N aqueous NaOH for 5 min, followed by water for 5 min and finally by separation buffer for 5 min.

2.5. Method B: Neutral capillary with electrokinetic injection

The electrophoretic separations were carried out using two eCAP-coated neutral capillaries, (i) 30 cm (20 cm effective length) × 50 μm i.d. × 360 μm o.d., and (ii) 30 cm (20 cm effective length) × 75 μm i.d. × 360 μm o.d., obtained from CS-Chromatographie (Langerwehe, Germany). An electrokinetic injection for 30 s was applied for introducing the sample into the capillary. The separation was performed using an applied current of −60 μA and a data acquisition rate of 8 Hz. Analytes were detected using direct UV absorbance at 210 nm. The capillary was conditioned by rinsing with water for 1 min and subsequently with running buffer (phosphate 50 mM, pH 6.5) for 1 min. Sample injections were made at the cathodic side of the capillary. The capillary was conditioned every day by rinsing it with water for 20 min before starting measurements and then with separation buffer for 5 min. The standard rinsing periods consisted of rinsing with water for one min followed by rinsing with buffer for one min. The capillary was stored in vials containing separation buffer inside the CE-instrument overnight after rinsing with water. For rinsing procedures we applied 40 psi of pressure for buffer and water. Under this standard rinsing protocol, the capillary could be used for several hundred runs.

2.6. Preparation of standard solutions for method validation

Stock solutions of nucleotides (dTMP, UMP and GMP, 10 mM) were prepared by dissolving the compounds in deionized water. Then they were further diluted to get 1 mM stock solutions of nucleotides in deionized water. Those were further diluted for the standard calibration curves in reaction buffer containing 20 mM Tris–HCl buffer, pH 7.4, 5 mM $MgCl_2$ and 1 mM ATP (concentration range 0.01–50 μM). UMP (20 μM) was used as an internal standard for method validation of dTMP, while cAMP was used as an internal standard for the method validation of GMP. Injections of standards were performed in triplicate. Calibration curves were obtained by plotting the corrected peak areas of the thymidine monophosphate (dTMP), aciclovir monophosphate (ACVMP) and ganciclovir monophosphate (GCVMP) peaks against their concentrations.

2.7. Investigation of the influence of reaction time on enzyme velocity

A set of experiments was performed in order to investigate whether the reaction proceeds linearly with time for a fixed concentration of substrate, using method A. The reaction mixture for the determination of the enzymatic velocity over different intervals of time contained: 2 mM thymidine, 50 mM Tris–HCl buffer, pH 7.4, 5 mM ATP and 5 mM $MgCl_2$. The reaction was started by adding 10 μl of TK solution (4 μg). The reaction was carried out at 37 °C and after various time intervals (5, 10, 15, 30, 60, 90, and 120 min) the reaction mixture was heated for 5 min at 99 °C using an Eppendorf Thermomixer Comfort to stop the reaction. Then 45 μl of the solution were transferred to a small CE vial containing 5 μl of an aqueous UMP solution (20 μM final concentration) as an internal standard. At each time interval three injections were made.

2.8. Effects of magnesium ions on the activity of thymidine kinase

Effects of different concentrations of 0.5, 1.0, 2.5, 5.0 and 10 mM of Mg^{2+} on the enzyme activity were monitored, using method B. The reaction mixture contained 1 mM ATP, 50 μM thymidine, 20 mM Tris–HCl buffer, pH 7.1, and different concentrations of $MgCl_2$. The reaction was started by adding 10 μl of suitably diluted TK (4 μg) and the mixture was incubated at 37 °C for 35 min, and then stopped by heating at 99 °C for 5 min. The dTMP formed was measured in duplicates by capillary electrophoresis in two separate experiments.

2.9. Capillary electrophoresis method for determination of kinetic parameters of HSV-1 thymidine kinase substrates

Kinetic parameters of HSV-1 TK substrates were determined by using CE method B. The enzymatic reaction was performed in a test tube according to a previously published method with modifications [14]. In a total volume of 100 μl, the reaction mixture containing 20 mM Tris–HCl buffer, pH 7.4, 5 mM $MgCl_2$, 1 mM ATP and different concentrations of the substrates (ACV, GCV, BVDU) ranging from 15.625 to 1000 μM, was brought to 37 °C (within 2 min). The reactions were initiated by adding 10 μl of HSV-1 TK (2–4 μg) and was then allowed to proceed at 37 °C for 15 min. The reaction was stopped by heating the mixture at 99 °C for 5 min. An aliquot of 45 μl of the reaction mixture was transferred to mini CE vials containing 5 μl of internal standard (UMP for BVDU, and cAMP for ACV and GCV, respectively). The final concentration of the internal standard in the assays was 20 μM. The absorbance at 210 nm was monitored continuously and the nucleotide concentrations were determined from the area under each absorbance peak. The experiments were repeated three times with triplicate injections. Control experiments were performed in the absence of substrate and in the absence of enzyme in order to take into account the spontaneous hydrolysis of ATP under the experimental conditions, which amounted to less than 1%. Kinetics for dT could not be determined due to the low K_m value which was below the detection limit.

2.10. Capillary electrophoresis method for the screening of compounds as ligands of HSV-1 TK

HSV-1 TK inhibition assays were performed by a modification of a published protocol [4]. Quantitative analysis was performed by CE, method B. Thymidine phosphorylation was measured in the absence and presence of ligand (aciclovir) at

three different concentrations of ligand (0.0625, 0.5 and 5 mM), while the concentration of thymidine was fixed at 100 μM. The reaction mixture contained 20 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$ and 1 mM ATP in a final volume of 100 μl. Mixtures were incubated at 37 °C for 2 min and then 4 μg of HSV-1 TK was added in order to initiate the reaction, similarly as for the substrate assay (see above). The reaction was stopped after 15 min by heating to 99 °C for 5 min.

3. Results and discussion

HSV-1 TK phosphorylates thymidine and other nucleosides and nucleoside analogs to their corresponding monophosphates. Capillary electrophoresis methods were optimized and validated for the determination of phosphorylated TK products and subsequently used for setting up a fast and easy CE-based TK assay. Several methods have previously been developed for the separation and quantitation of nucleosides and nucleotides after enzymatic reactions [26–31]. Theses studies were taken as a starting point for adaptation to the biological project of monitoring HSV-1 TK. Subsequently, the methods were substantially improved and optimized with regard to sensitivity, precision, and duration of analyses.

3.1. Fused-silica capillary with pressure injection (method A)

Initially, a fused-silica capillary was used for the separation of nucleosides and nucleotides. Sodium phosphate buffer (20 mM) was selected as the running buffer as it does not show UV absorption between 200 and 300 nm and therefore does not interfere with the UV detection of nucleosides and nucleotides [32]. Absorption at 260 nm was selected for quantitation. The low concentration of 20 mM phosphate buffer was favorable since higher concentrations of the running buffer generated high currents and excessive Joule heating. The most important parameter affecting electrophoretic separation of weak electrolytes is the pH value of the running buffer. In order to achieve a high resolution of the compounds and a high reproducibility of migration times, a pH of 8.5 was found to be optimal. Another critical parameter of the method affecting reproducibility of migration times is the applied current: assays were performed at a constant current of 95 μA. Using an uncoated fused-silica capillary and normal polarity, the eluting sequence was: nucleosides followed by monophosphates, diphosphates, and finally triphosphates. The product dTMP eluted after less than 7 min, while ATP eluted after less than 11 min. A typical electropherogram is shown in Fig. 2; in that experiment the effects of different incubation times (15 min, Fig. 2A, and 30 min, Fig. 2B) on the conversion of thymidine to TMP was investigated. Due to variations in the hydrodynamic injection of small volumes (nanoliters) the peak area of UMP is different in Fig. 2A and B, but such variations are compensated for by using an internal standard.

Method A was used initially to optimize some parameters for the enzymatic reaction. However, a major drawback was the observation that the silanol groups on the capillary surface interacted with the positively charged protein (enzyme) resulting in a poor reproducibility of migration times, a loss of efficiency in separations resulting in peak broadening, and a decrease in sensitivity. Therefore, an alternative method (B) was developed in which a polyacrylamide-coated capillary was used.

3.2. Neutral capillary with electrokinetic injection (method B)

The application of coated capillaries instead of fused-silica capillaries has been found to be advantageous for biological applications, since adsorption of proteins and analytes to the capillary wall can be avoided [33,34]. Ucon-coated and polyacrylamide-coated fused-silica capillary capillaries have previously been used for the separation of nucleotides [27,35,36]. It was found that the electroosmotic flow in these capillaries was significantly reduced and coating was inert to biological samples [27,37]. Therefore, we investigated the use of polyacrylamide-coated capillaries for the monitoring of the TK reaction and for performing TK assays. The separations were optimized with respect to efficiency, selectivity, and running time. A high resolution was obtained using sodium phosphate buffer pH 6.5, in which the nucleotides were stable, while lower pH values may result in hydrolysis [37]. The polyacrylamide coating is very stable at acidic or neutral conditions [33], whereas buffers with higher pH values shorten the life-time of the capillaries.

The ionic strength of the running buffer is an important factor to control the separation efficiency and migration times. A 50 mM concentration of the running buffer was selected, since lower concentrations gave poor resolution, while higher concentrations increased the migration time without further improvement in the peak separation efficiency. A short capillary length (30 cm (20 cm effective length)) was used in order to reduce the analysis time and for improving the rinsing cycles, because in shorter capillaries more rinsing solution can be passed through within the same period of time. Using a coated capillary the overall rinsing time was reduced as compared to fused-silica capillaries. The eluting sequence was triphosphates, diphosphates, followed by monophosphates (see Fig. 3). Reproducibility of migration time in coated capillaries was significantly higher than in untreated fused-silica capillaries as shown in Table 1.

Sample preconcentration has been used to increase the sensitivity of analytical assays. Special detectors, such as laser-induced fluorescence and electrochemical detectors, are another solution for the problem of the low sensitivity of CE. However, another simple alternative is sample stacking. It is very convenient to utilize the electrical current not just for separation but also for sample concentration directly in the capillary [38,39]. Therefore, electrokinetic injection was used to increase the sensitivity by on-line preconcentration as compared to the hydrodynamic injection. Electrokinetic injection, within the same amount of time, gives about four times higher amounts of compounds injected as compared to hydrodynamic injection [38]. In contrast to pressure injection, which leads to the injection of all compounds present in a solution, with electrokinetic injection only negatively charged molecules are injected. Therefore, molecules present in the assay solution that may interfere

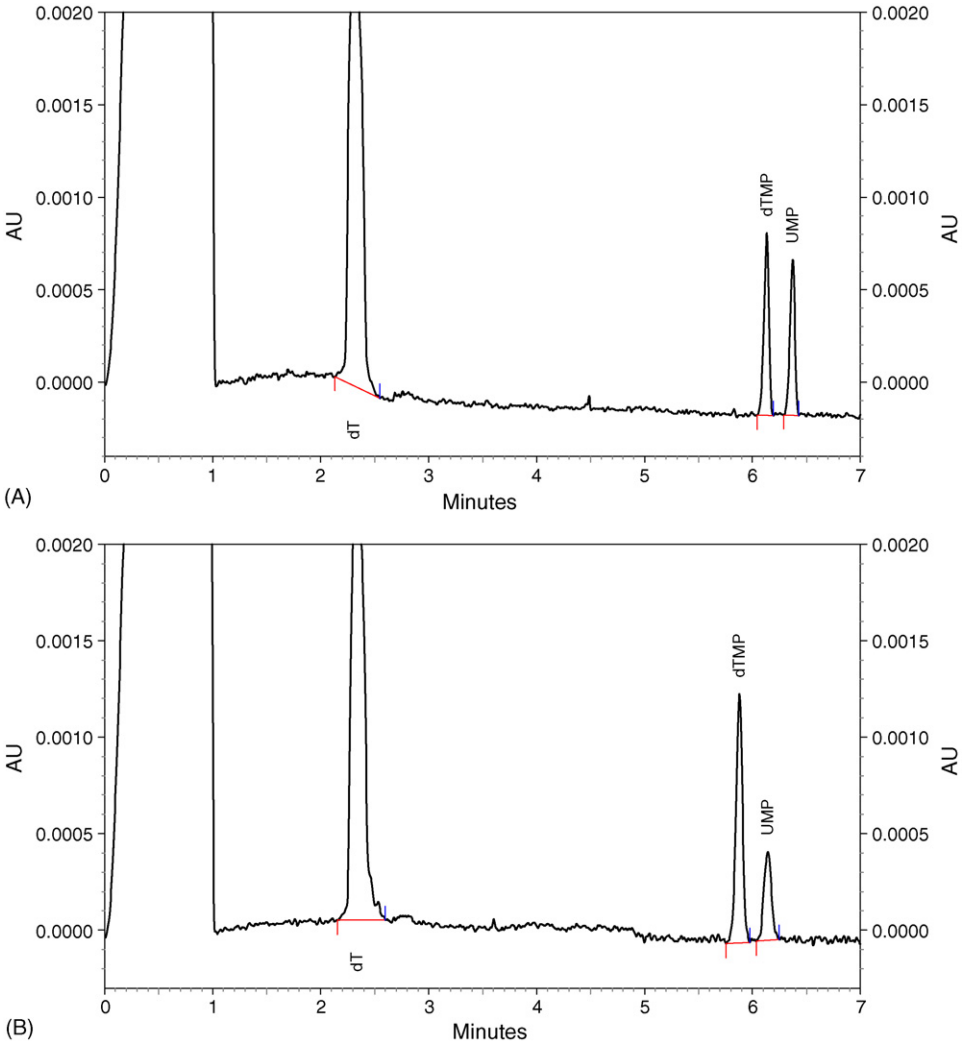


Fig. 2. Typical electropherograms for the investigation of the effects of different reaction times on the phosphorylation rate of dT to dTMP by HSV-1 TK using CE method A. The concentration of dT was 2 mM in enzyme assay buffer (50 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 5 mM ATP, containing 4 μg of enzyme per tube in a volume of 100 μl). UMP (20 μM) was used as an internal standard. The separation conditions were: 20 mM potassium phosphate buffer, pH 8.5, fused-silica capillary, 40 cm length (30 cm to the detector), 75 μm i.d., 95 μA, detection at 260 nm, pressure injection with 0.1 psi for 25 s and normal polarity. ADP (8.1 min) and ATP (10.5 min) migrated after UMP, and are not shown in the electropherogram. The incubation time was 15 min (A), and 30 min (B).

with the determination are excluded. This offers the possibility to detect the nucleotides at 210 nm where the absorption is higher than at the wavelength of 260 nm used in method A, and the sensitivity can thus be increased. Another approach to increase the sensitivity would be to increase the capillary internal diameter. Therefore, a 75 μm internal diameter capillary was tried out. However, the precision of migration time was reduced with the larger internal diameter of the capillary (Table 1). The limits

Table 1
Method validation parameters: limits of detection and quantitation, precision of migration time and linearity of the standard calibration curve

Compound	dTMP			GMP
	50 μm NC[a]	75 μm NC[a]	75 μm FC[b]	50 μm NC[a]
Limit of detection (μM) ± S.D.[c]	0.36 ± 0.20	1.03 ± 1.4	2.60 ± 0.60	0.86 ± 0.13
Limit of quantitation (μM) ± S.D.	1.08 ± 0.20	3.11 ± 1.4	6.80 ± 0.60	3.31 ± 0.13
Correlation coefficient; R^2	1.000	0.990	0.970	0.999
Mean value of migration time (min) ± S.D. (n = 12)	6.11 ± 0.01	7.96 ± 0.042	5.85 ± 0.15	6.34 ± 0.02
R.S.D.[d] of migration time (%)	0.16	0.53	2.56	0.32

[a] NC, neutral capillary.
[b] FC, fused-silica capillary.
[c] S.D., standard deviation.
[d] R.S.D., relative S.D.

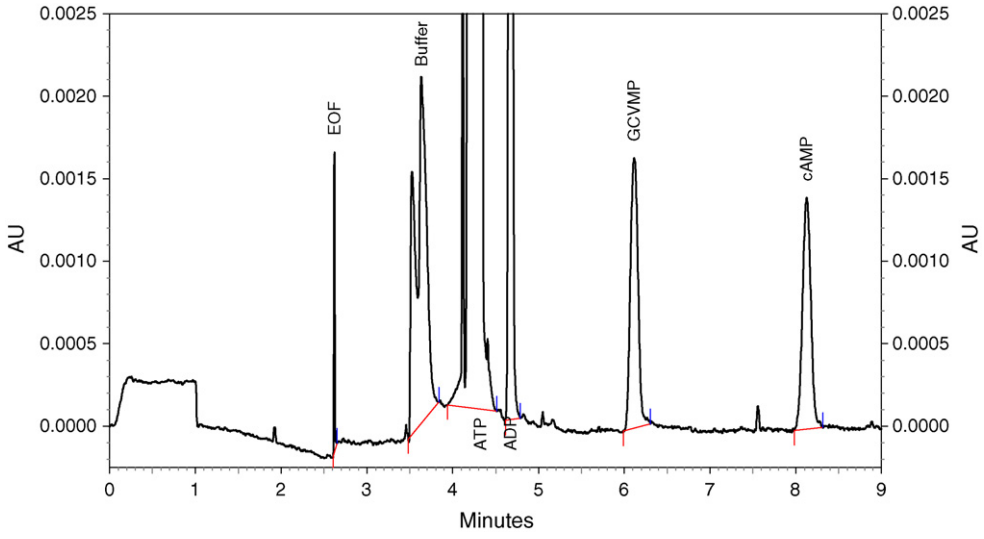


Fig. 3. Typical electropherogram for the determination of the K_{cat} value for ganciclovir (GCV) using CE method B. The concentration of GCV was 500 μM in enzyme assay buffer (20 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM ATP containing 4 μg of enzyme per tube in a volume of 100 μl with a final concentration of 1% DMSO). The incubation time was 15 min. Cyclic adenosine monophosphate (cAMP, 20 μM) was used as an internal standard. Due to the application of electrokinetic injection the neutral molecule GCV was not injected into the capillary. The separation conditions were: 50 mM potassium phosphate buffer, pH 6.5, neutral capillary, 30 cm length (20 cm to the detector), 50 μm i.d., −60 μA, detection at 210 nm, electrokinetic injection with −6 kV for 30 s and reverse polarity.

of detection and quantitation by both, hydrodynamic injection using method A, and electrokinetic injection using method B are compared in Table 1.

A CE-based assay for the determination of TK and thymidylate kinase activity has recently been described [24]. However, the present study is the first report on a CE-based assay for the characterization of various, structurally diverse TK substrates and inhibitors. In the published method [24] the limit of detection of dTMP was 2.6 μM using acetonitrile-salt sample stacking. In our CE-based assays, the detection limit for dTMP was much lower (0.36 μM) using a simple eletrokinetic sample stacking method. The method was optimized with regard to injection time and applied voltage. Longer injection times gave low reproducibility while very short injection times decreased the sensitivity. Different voltages and injection times, e.g. −10 kV for 5 s, −6 kV for 15 s, and −6 kV for 30 s, were used with the short capillary. The highest sensitivity and reproducibility was obtained with −6 kV for 30 s.

3.2.1. Selection of the assay buffer for the enzymatic reaction

In the next step, the assay buffer concentration was optimized for electrokinetic injection. Two concentrations, 20 and 50 mM Tris–HCl buffer, pH 7.4, were investigated; no difference in TK activity was found with both buffers. Chloride ions and other electrolytes in the buffer have faster mobility and thereby concentrate ahead of the analytes at the tip of the capillary thus decreasing the field strength and consequently decreasing the amount of analyte being injected. Thus, 20 mM Tris–HCl buffer, pH 7.4 was selected for the assay, because low concentration of the sample buffer improves the sensitivity in electrokinetic injection [38–40]. TK is a magnesium-dependent enzyme. The optimal concentration was determined to be 5 mM magnesium chloride. Higher concentrations gave broad peak shapes and poor resolution, while lower concentrations resulted in low catalytic activity.

3.2.2. The use of an internal standard

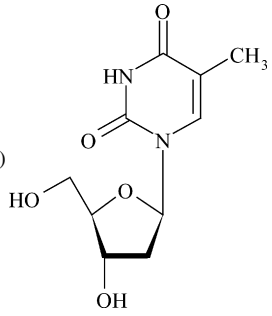
The use of an internal standard (IS) has been generally accepted to be crucial for reproducibility in CE to compensate for injection errors and minor fluctuations of the migration times. The major source of imprecision remaining when using commercial CE instrumentation is injection volume variability. Therefore, the use of an IS improves precision of quantitation [41]. An ideal IS is water-soluble, and UV-detectable at 210 nm. Moreover, the migration time of the IS should differ from the migration times of the reaction compounds and not prolong the total analysis time. UMP, being UV-detectable at 210 nm, was chosen as an internal standard for the determination of the kinetic parameters of BVDU with a migration time of less than 8 min. However, UMP could not be used as an internal standard for the determination of ACV and GCV because the phosphorylated products of ACV and GCV comigrated with UMP. Therefore, cAMP was selected as an IS for the analysis of the TK reaction with ACV and GCV.

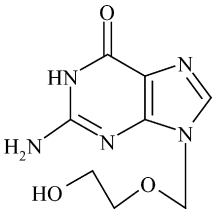
3.3. Validation of the capillary electrophoresis methods

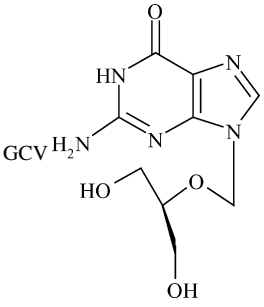
3.3.1. Limits of detection, quantitation and linearity

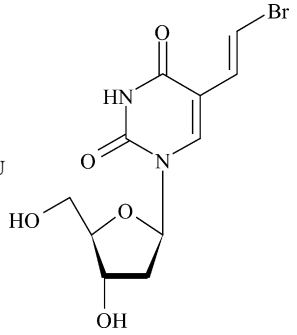
The developed CE methods A and B for monitoring the concentrations of phosphorylated products of TK were validated. Three different capillaries with different internal surfaces or diameters were used for the determination of limits of detection, quantitation and linearity of dTMP. Validation parameters for GMP were obtained only with a neutral capillary of 50 μm i.d., which had proven to be the capillary of choice. Since the phosphorylated product of aciclovir, aciclovir monophosphate, and of ganciclovir, ganciclovir monophosphate, were not available

Table 2
Kinetic analysis of substrates of HSV-1 thymidine kinase

Thymidine (dT) 

ACV 

GCV 

BVDU 

Substrate	Capillary electrophoresis[a]		Literature values	
	K_m (μM)	K_{cat} (s^{-1})	K_m (μM)	K_{cat} (s^{-1})
dT	n.d[b]	n.d[b]	0.2 [45][c], 0.38 [47][d], 0.9 ± 0.08 [48][d]	0.44 [46][c], 0.35 ± 0.004 [11][d]
ACV	238 ± 94	0.011 ± 0.002	200 [45][c], [11][d], 417 [47][d]	0.015 [10][d], 0.13 [46][c]
GCV	27.2 ± 18.6	0.12 ± 0.05	69 ± 4 [48][d], 47.6 [47][d], 48 [46][c]	0.102 [10][d], 0.10 [46][c]
BVDU	25.3 ± 13.4	n.d[e]	50 [13][f]	n.d[g]

[a] $n = 3$.
[b] Could not be determined due to low K_m value and limited sensitivity of the CE assay method.
[c] Determined by standard spectrophotometric assays.
[d] Determined by standard radiometric assays.
[e] Could not be determined due to unavailability of isolated reaction product BVDU-MP for measuring a calibration curve.
[f] Determined by high-performance liquid chromatography.
[g] Literature value was not available.

for determining calibration curves, we constructed the calibration curve using the structurally related GMP, which contains the identical chromophore (guanine partial structure, for structures see Table 2). The other parts of the molecules do not contribute to their UV absorption.

CE employing a neutral capillary with electrokinetic injection gave lower detection limits as compared to a fused-silica capillary with pressure injection. The highest sensitivity was obtained with the polyacrylamide-coated capillary of 50 μm internal diameter (dTMP: LOD 0.36 μM, LOQ 1.08 μM, see Table 1). The neutral capillary of 75 μm internal diameter gave somewhat higher values (LOD 1.03 μM, LOQ 3.11 μM), while with the fused-silica capillary (75 mm outer diameter) and pressure injection the lowest sensitivity was observed (LOD 2.60 μM, LOQ 6.80 μM). The 75 μm (i.d.) polyacrylamide-coated capillary showed an unexpectedly high detection limit along with an increased standard deviation of migration time. It could be reasoned that with a large i.d. electrokinetic injection could inject more buffer ions and other species into the capillary and thus result in a more unfavorable detection and quantitation limit. This might be the reason for the fact that 75 μm (i.d.) coated capillaries are rarely used and only provided by few companies. The regressive linearity coefficients for dTMP obtained with the three capillaries were 1.000 (50 μm (i.d.) neutral capillary), 0.990 (75 μm neutral capillary), and only 0.970 for the 75 μm fused-silica capillary. Measurements with the 50 μm (i.d.) polyacrylamide-coated capillary showed linearity of the calibration curve in the range from the lowest determined concentration to the highest concentrations measured in the enzyme assays. For dTMP using the 50 μm (i.d.) polyacrylamide-coated capillary the determined equation was $y = 40980x - 8254$ ($R^2 = 1.000$) and the relative standard error of the slope was 2.02%. The limit of detection and quantitation for GMP with the same capillary, which had given best results for dTMP, was 0.86 μM (LOD), and 3.31 μM (LOQ), respectively. The data and standard deviations are collected in Table 1.

3.3.2. Precision of migration time

The precision of migration time was calculated by injecting dTMP and GMP solutions in separation buffer for 12 times. In the presence of an IS, the relative standard deviations (R.S.D.) of migration time were calculated to be 0.16% (50 μm coated capillary), 0.53% (75 μm coated capillary) and 2.56% (75 μm fused-silica (Table 1). Thus, high precision was found with coated capillaries, while it was lower for untreated capillaries. The reason for the good precision and reproducibility in migration times for neutral capillaries is due to the absence of an EOF. In addition, the coated capillaries are inert to biological samples, which further increases the precision of the analysis.

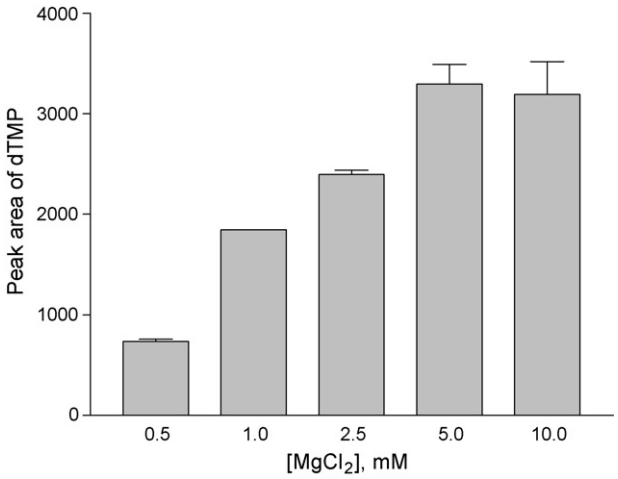


Fig. 4. Effects of different $MgCl_2$ concentrations on the activity of thymidine kinase at a fixed concentration of ATP, determined by capillary electrophoresis. Determinations were performed at pH 7.4 using 1 mM ATP, 50 μM thymidine and various concentrations of $MgCl_2$ (0.5 to 10 mM). The separation conditions were 50 mM phosphate buffer, pH 6.5, neutral capillary, 30 cm length (20 cm to the detector), 50 μm i.d., 60 μA, 25 °C, detection at 210 nm, electrokinetic injection for 30 s at −6 kV. Data points represent means ± S.D. from two separate experiments each run in duplicate.

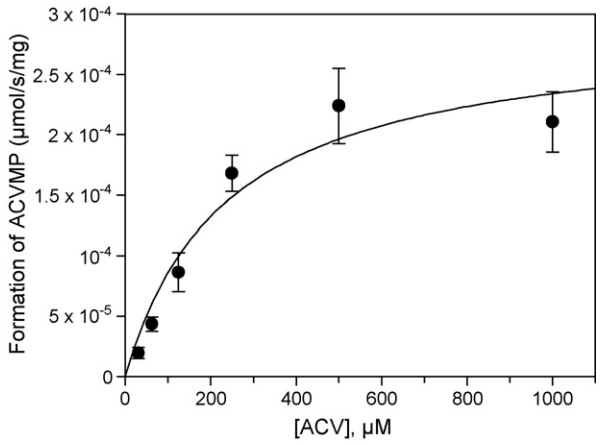


Fig. 5. Michaelis-Menten plot of initial ACV concentration with respect to the reaction velocity (μmol of ACVMP produced in one second per mg of enzyme) for the determination of K_m and K_{cat} values by capillary electrophoresis, using method B. For CE conditions see Fig. 3. The assay buffer was 20 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM ATP containing 4 μg of enzyme per tube in a volume of 100 μl and various concentrations of ACV. Data points represent means ± S.D. from three separate experiments each run in duplicate. For determined K_m and K_{cat} values see Table 2 (ACV = aciclovir, ACVMP = aciclovir monophosphate).

3.4. Biological assays

Preliminary biological studies regarding the reaction time (see Section 3.4.2) were performed using method A (fused-silica capillary) while all other biological assays were conducted with the optimized method B (polyacrylamide-coated capillary of 50 μm i.d., electrokinetic injection).

3.4.1. Magnesium-dependence of enzymatic reaction

The activity of TK as a function of various concentrations of $MgCl_2$ was examined. Mg^{2+} is a co-substrate of the enzyme. TK showed maximal activity at a concentration of 5 mM of $MgCl_2$. After increasing the concentration of $MgCl_2$ above this limit, there was no further increase in enzymatic activity, whereas lower concentrations resulted in reduced activity (Fig. 4). TK did not show any activity in the absence of magnesium, thus it showed an absolute requirement for magnesium. The dependence of TK activity on Mg^{2+} and ATP indicates that the ATP-Mg complex is the true substrate for the enzyme [42,43].

3.4.2. Reaction time

The influence of the reaction time on enzyme velocity was determined. The peak area of dTMP increased linearly with incubation time up to an incubation time of 120 min. A reaction time of 15 min was selected for the TK enzyme assays. Typical electropherograms for the investigation of the influence of the reaction time on the product formation by HSV-1 TK are shown in Fig. 2A and B, where reaction times of 15 and 30 min are compared.

3.4.3. Michaelis-Menten analysis of HSV-1 TK substrates

A selection of substrates was phosphorylated to the corresponding monophosphates by HSV-1 TK, and the progress of the reaction was monitored by capillary electrophoresis. The K_m and K_{cat} values were calculated by fitting the initial reaction rates for the formation of product (phosphorylated substrate) as a function of substrate concentration into the Michaelis-Menten equation. For ACV, a K_m of 238 μM ($n = 3$, S.D. = 94) was determined (see Table 2). Fig. 5 shows the Michaelis-Menten plot of the phosphorylation of ACV by HSV-1 TK. Further K_m values thus obtained were 27.2 μM for GCV ($n = 3$, S.D. = 18.6) and 25.3 μM for BVDU ($n = 3$, S.D. = 13.4) (Table 2). Due to the limited sensitivity of the assay, the low K_m (in the nanomolar range) and K_{cat} values of dT could not be determined. Although the Michaelis-Menten constants (K_m) for HSV-1 TK substrates obtained in different studies are not always comparable due to different analytical methods, reaction conditions, and enzyme sources used [10] our results were in the same range as the reported values obtained by spectrophotometric and radiometric assays for ACV [11,44,45], GCV [10,44] and BVDU [13] (see Table 2). An increased (K_{cat}/K_m) for the substrate towards the natural substrate dT correlates well with enhanced production of the active, phosphorylated drug within the cell [46]. For the determination of the V_{max} values of the guanine derivatives ACV and GCV, a calibration curve was obtained with GMP, which has no significant difference in molar UV absorption as compared to the phosphorylated substrates of ACV and GCV. The determined K_{cat} values were 0.011 ($n = 3$, S.D. = 0.002) for ACV and 0.12 ($n = 3$, S.D. = 0.05) for GCV (Table 2). The K_{cat} value of BVDU could not be determined due to the unavailability of isolated reaction product (BVDU-MP) for determining a calibration curve. However, the K_m value could be calculated from the added concentration of BVDU (Table 2). K_{cat} like K_m values determined by the new CE method were well in accordance with published data (see Table 2).

Table 3
Inhibition of the thymidine kinase reaction by aciclovir determined by capillary electrophoresis (CE)[a]

Concentration of ACV (mM)	Phosphorylation of dT (%)[b] ($n = 3$)[c]
0 (control)	100 ± 0
0.0625	97 ± 5.4
0.5	71 ± 1.6
5.0	62 ± 0.8

[a] Data were collected in 20 mM Tris buffer, pH 7.4, containing 5 mM $MgCl_2$, 1 mM ATP and 100 μM thymidine, in a final volume of 100 μl. Reactions were incubated at 37 °C for 2 min and then 4 μg of HSV-1 TK was added in order to initiate the reaction; the incubation time was 15 min. The reaction was stopped by heating to 99 °C for 5 min. For CE conditions, see Fig. 3 (CE method B).
[b] Percentage of phosphorylated product obtained without ACV was set at 100%.
[c] All values are means ± S.E.M. from three separate experiments.

3.4.4. Enzyme inhibition assay

ACV is an alternative substrate for HSV-1 TK, and therefore it is also a competitive inhibitor of dT phoshorylation. It was selected for performing enzyme inhibition assays by CE. The results are presented in Table 3. The reactions were performed with varying ACV concentrations (up to 5 mM) while the substrate, thymidine, was kept constant at a high concentration of 100 μM (ca. 200-fold K_m value). A lower concentration of dT could not be used due to the limited sensitivity of the CE assay with regard to the determination of the product dTMP. The amount of HSV-1 TK protein was 4 μg in a total assay volume of 100 μl. As the concentration of ACV increased, the percent formation of dTMP decreased, as shown in Table 3. In the presence of 0.5 and 5 mM of ACV the dTMP formation was reduced by 29 and 38%, respectively. These results show that the CE assay cannot only be used to monitor TK substrates, but is also suitable to investigate enzyme inhibitors.

4. Conclusions

We developed a simple, fast and sensitive method for the investigation of substrates of HSV-1 TK, some of which are used as antiviral drugs, for the determination of their Michaelis-Menten (K_m) constants and catalytic constants (K_{cat}). The new CE method can also be used for the investigation of HSV-1 TK inhibitors and the screening of compound libraries in the search for novel inhibitors and alternative substrates. The phosphorylated products of the HSV-1 TK reaction could be successfully separated from the substrates using a coated capillary with electrokinetic injection. The new CE method has multiple advantages in comparison with standard TK assays, such as no need for expensive radiolabeled substrates and a minimal use of reagents. In addition, the assay is fully automated, allows the use of 96-well plates, and can be carried out in less than 10 min. In comparison to the CE-based assay for the determination of TK and thymidylate kinase activity [24] that has recently been described, our method has clear advantages, such as increased sensitivity, no requirement of sample pretreatment before CE analysis, and no requirement of long rinsing procedures which are typical for the use of fused-silica capillaries in biological assays.

Acknowledgements

We thank Christine Klötzer for the expression, purification, and characterization of HSV-1 TK during her stay at the ETH Zürich as an undergraduate exchange student.

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