Original Article

Facile method for determination of deoxycytidine kinase activity in biological milieus

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Wei-Hua Hao [a,c], Li-Chieh Yang [a], Jong-Jing Wang [a], Chang-Shan Hsu [a],
Li-Chien Chang [b], Kuang-Yang Hsu [c,*]

[a] InnoPharmax Inc., Taipei, Taiwan, ROC
[b] School of Pharmacy, National Defense Medical Center, Taipei, Taiwan, ROC
[c] School of Pharmacy, Taipei Medical University, Taipei, Taiwan, ROC

ABSTRACT

A new analytical method for determining deoxycytidine kinase (dCK) activity in biological milieus using luminescence is reported here. This method, based on utilizing adenosine triphosphate (ATP) as the sole phosphate donor in the kinase reaction and monitoring ATP consumption via a luciferase-based chemiluminescence reaction, is capable of detecting dCK activity without the use of specific substrates or radioisotope techniques. Comparing with the reverse-phase high-performance liquid chromatography method, this new method is suggested to be efficient and sensitive. Further, application of the proposed method for profiling dCK activity in cultured cancer cells revealed that a cervix cell line exhibited the highest dCK activity to gemcitabine metabolism.

1. Introduction

Deoxycytidine kinase (dCK) is an enzyme expressed in eukaryotic cells [1,2] that catalyzes phosphate transfer between adenosine triphosphate (ATP) and 2′-deoxynucleosides. It plays an essential role in the salvage pathway of nucleotide metabolism. Therapeutically, dCK phosphorylates anticancer nucleoside analogs such as gemcitabine (2′,2′-difluorodeoxycytidine) [3] and 2-chlorodeoxyadenosine (CdA) to their cytotoxic forms [4], which are effective chemotherapeutic agents for some solid tumors and leukemia. Clinically, dCK is reported to play a role in mediating the effectiveness of nucleoside antimetabolite therapies [5—7].

Cumulative pharmacological and pharmacogenetical data indicate that dCK activity correlates with both efficacy of gemcitabine therapy and resistance to the drug [5—10]. The dCK overexpression increased the sensitivity of colon, breast, and lung cancer cells to gemcitabine [7,11]; by contrast, dCK downregulation enhanced acquired drug resistance in pancreatic cells [6,11]. Levels of dCK protein expression in human pancreatic cancer tissues are correlated with the overall survival of the gemcitabine-treated patients [10,12]. The combined evidence suggests that dCK is a versatile biomarker in nucleoside anticancer therapy and governs the effectiveness of gemcitabine treatment.

Only a few methods have been established for detecting dCK in biological samples. Antibody-based methods such as western blotting and enzyme-linked immunosorbent assay have limited ability to detect the active form of dCK; therefore, antibodies that can recognize various dCK substrates are

required [13,14]. Conventional assays for dCK, based on the use of radioactive substrates such as [8-3H] CdA and a series of binding and washing procedures to separate "hot" and "cold" portions to assess activity [4,14], are complicated with the use of radioisotope materials, which involves problems such as waste disposal and radiation safety. Bierau et al [15] developed a high-performance liquid chromatography (HPLC) method using nonradiolabeled CdA with inherent limitation in sensitivity because nucleosides have no specific chromophores for spectrophotometric detection. Therefore, in order to explore impacts of dCK in pharmacological and clinical studies, development of an efficient and sensitive method to determine dCK activity in biological milieus is mandatory.

ATP has been found to serve as the phosphate donor in the dCK reaction, and ATP analysis is correlated to detect dCK activity enzymatically (Fig. 1). Several highly sensitive fluorescence and luminescence methods have been developed for the discovery of kinase inhibitors in preclinical studies [14]. We accordingly developed an efficient luminescent assay for determining dCK activity in biological milieus. The assay is based on the use of anion exchange beads to purify dCK from complex biological samples and a luciferase-based ATP assay to quantitate dCK activity. With ATP serving as the sole phosphate donor to dCK substrates in the proposed assay

reaction (Fig. 1), ATP consumption, monitored by a luciferase-based chemiluminescent reaction, can be utilized to determine the enzymatic activity of dCK. In the reaction, the luminescent signal generated from the conversion of beetle luciferin to oxyluciferin with the stoichiometry of one ATP molecule to one photon per turnover is inversely correlated to the dCK activity. This new method has been validated with comparison to the conventional HPLC method. Its application in profiling dCK activity in cancer cells has also been evaluated.

2. Methods

2.1. Materials

Deoxycytidine and deoxycytidine triphosphate (dCTP) were purchased from Sigma-Aldrich (St Louis, MO, USA). Gemcitabine (2′,2′-difluorodeoxycytidine) was obtained from Innopharmax (Taipei, Taiwan). Kinase-Glo reagent was purchased from Promega (Madison, WI, USA). Other chemicals were of analytical grades and obtained from Sigma-Aldrich. Milli-Q water prepared by a Millipore system was used in buffer preparation.

2.2. Cell lines

HeLa, HCT116, LX-1, 780-O, and MCF-7 cells were obtained from the American Type Culture Collection (10801 University Boulevard Manassas, VA 20110 USA). In brief, HeLa and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), HCT116 and 786-O cells were cultured in RPMI-1640, and LX-1 cell was cultured in Minimum Essential Medium. All media were supplemented with 10% Fetal Bovine Serum (FBS); and cells were maintained at 37°C in 5% CO_2 and routinely passaged every 2–3 days in a 75T flask.

2.3. Protein extraction and quantitation

Cell pellets suspended in extraction buffer (PRO-PREPTM Protein Extraction solution; iNtRON, Kyunggi-do, Korea) were sonicated twice on ice for 10 seconds each. After sonication, extracts were incubated at −20°C for 10 minutes and centrifuged at 13,628g at 4°C for 10 minutes. Supernatants containing the cellular proteins were collected and maintained at −20°C prior to analysis.

Protein concentrations of the cell extracts were determined by a Pierce 660 nm Protein Assay (Thermo, 81 Wyman Street, Waltham, Massachusetts 02454, USA) using bovine serum albumin as the protein standard. The calibration curve was constructed by plotting the optical densities obtained at 660 nm versus the individual known bovine serum albumin concentration ranging from 0.1 mg/mL to 2.0 mg/mL.

2.4. Developed luminescent assay

To distinguish dCK from other kinases that may interfere with the assay, a quick purification procedure adapted by Quan et al [16] was employed. In brief, quantitated cell extracts containing dCK were incubated with 0.5 mL HiTrap Q
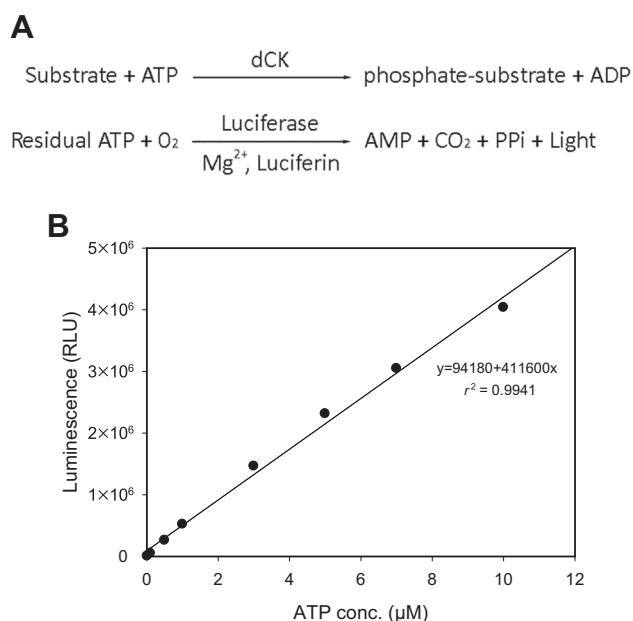

Fig. 1 – (A) Reaction scheme of the luminescence dCK assay. Given that the phosphate transfer reaction consumes one ATP and one oxygen molecule to produce one photon of light in the luciferase-based system, the light measured as RLU is correlated with the amount of residual ATP and inversely reflects dCK activity. (B) A linear relationship is observed between the luminescence signal and ATP concentration. The correlation coefficient (r^2) is 0.9941 for ATP in the concentration range of 0.1–10μM. ADP = adenosine diphosphate; ATP = adenosine triphosphate; dCK = deoxycytidine kinase; RLU = Relative light unit.

beads (Amershan Bioscience, Uppsala, Sweden) in a 1.5 mL vial for 1 minute. After binding of dCK to the ion-exchange beads, the beads were washed with 0.01 M Tris-HCl buffer (pH 7.0, containing 5 mM β-mercaptoethanol) three times to remove possible interference proteins. Subsequently, dCK was eluted by the same Tris-HCl buffer containing 0.5 M NaCl (0.5 mL) and subjected to the developed luminescent assay.

To reduce interference from other coeluted proteins, each sample was divided into two equal parts for the determination of luminescence signal in the presence or absence of dCK substrate in the assay. Each sample was incubated with 10 μM of each dCK substrate (deoxycytidine and gemcitabine) and ATP in a 25 μL reaction buffer at 25°C for 0–15 minutes for the ATP consuming assay. At each time point, an equal volume of Kinase-Glo reagent was added to quench the reaction and develop the luminescent signal simultaneously at 25°C for 10 minutes. The luminescent signal was recorded using a luminescence microplate reader (Infinite; Tecan, Männedorf, Switzerland) with the integration time set at 0.25 seconds according to the manufacturer's instruction, and the ATP concentrations detected between 0 minutes and 4 minutes were used to calculate the dCK activity after subtracting nonspecific basal activity, as determined from the blank sample used as a control. The dCK activity was expressed as nmole/min/mg protein.

Validation of the developed method was performed using the linearity, intra- and interday variations, and specificity. Each sample was analyzed five times using the proposed method, and the limit of detection and limit of quantification were calculated as the sample concentration generating a three- and 10-fold larger luminescence signal than the baseline noise, respectively.

2.5. Comparative HPLC method

Biological samples used in the comparative HPLC analysis [15] were the same as those used in the developed luminescence assay. In brief, after the kinase reaction was performed following the above procedures, the samples were quenched with an equal volume of cold methanol on ice for 10 minutes. The cold methanolic sample solution was centrifuged at 11,612g at 4°C for 10 minutes, and the supernatant was collected and subjected to HPLC analysis. HPLC runs were conducted on Waters Model 2695 HPLC system equipped with an Hypersil OSD-2 column (250 mm × 4.6 mm ID, 5 μm particle size) and a variable-wavelength UV detector set at 275 nm. The mobile phase consisted of phosphate buffer (0.1 M, pH 2.4–2.6) and 2.5 mL 85% phosphoric acid. The injection volume was 50 μL, and the flow rate of mobile phase was 1.5 mL/min.

2.6. Statistical analysis

All experiments were repeated at least three times. Results were expressed as mean ± standard error of the mean. Statistical significance of differences was assessed by a t test, and $p < 0.05$ was considered significant.

3. Results

3.1. Development and validation of the proposed dCK assay

Fig. 1 shows the reaction scheme of the proposed coupled enzyme assay. The measured chemiluminescence was related directly to the residual ATP, which inversely corresponds to dCK activity. Eight known concentrations of ATP solution were used to establish the linearity of the generated signal. Highly linear relationship of ATP between 0–10 μM were obtained for the developed dCK assay as the basis of ATP working concentration with the limit of detection and limit of quantification of 0.016 μM and 0.048 μM, respectively.
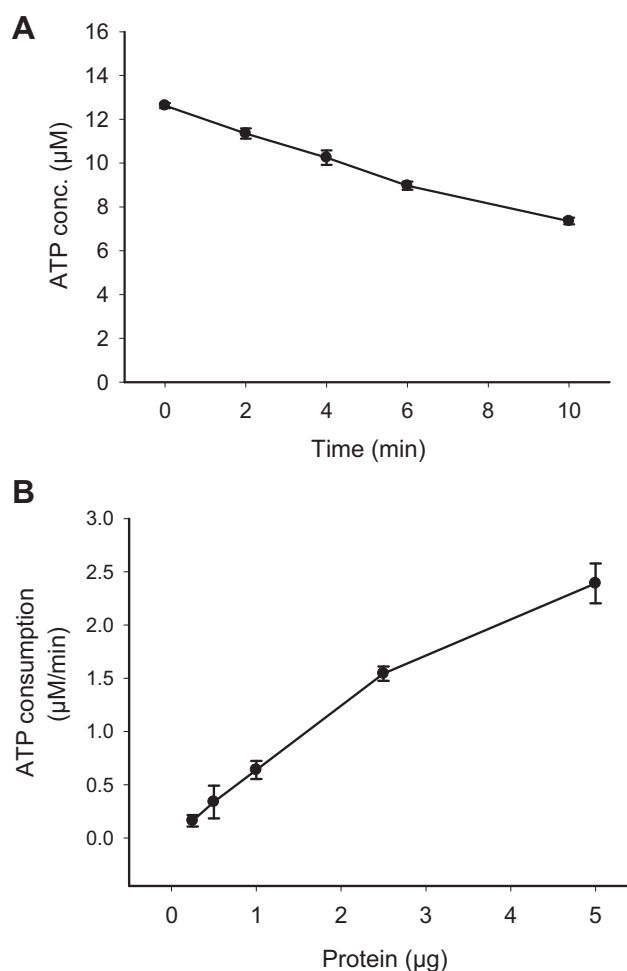
Fig. 2 — (A) Time dependence of the dCK reaction. One microgram of the dCK-containing sample from rat tissue (small intestine) was incubated with 10μM gemcitabine and 10μM ATP. The data for each time point represent the mean of five measurements. (B) ATP consumption versus dCK activity. Various dCK-containing samples (i.e., 0.25 μg, 0.5 μg, 1 μg, 2.5 μg, and 5 μg) were used to validate the quantitation relationship. In general, the specific activity of dCK was calculated on the basis of ATP consumption between 0 minutes and 4 minutes. ATP = adenosine triphosphate; dCK = deoxycytidine kinase.

The feasibility of the developed method for biological samples was demonstrated using dCK-containing samples obtained from rat tissue (small intestine). Based on the stoichiometry between ATP and dCK substrate such as gemcitabine, Fig. 2A illustrates that a quantifiable relationship between ATP consumption and dCK activity was obtained when the sample was incubated with an exogenous dCK substrate and ATP. With the ATP consumption by dCK-containing sample (1 μg) corresponding with dCK activity after subtracting the basal ATP consumption in the blank sample, which was assayed without adding a dCK substrate, dCK activity (nmole/min/mg protein) was calculated easily on the basis of the ATP consumption rate in the test period (i.e., 10 minutes). Based on a 4-minute kinetic measurement, the consumption rate was positively correlated to the amount of protein added in the range of 0.25−5 μg (Fig. 2B). As the established relationship was clearly a reflection of kinase activity, it is evident that the developed method can be used to determine dCK activity in a substrate-specific manner.

To further verify the specificity of the developed assay, the dCK inhibitor dCTP was employed, and it functions as a feedback competitor with dCK substrates such as gemcitabine for DNA polymerase [3,16]. As shown in Fig. 3, ATP consumption in the dCK-containing sample was inhibited and dependent on the various concentrations of dCTP added to the assay milieus, and 200 μM dCTP inhibited dCK activity by approximately 70% in a 1 μg dCK-containing sample. These results suggested that the developed method depicted properly a quantifiable relationship between luminescence signals and dCK activity in biological homogenates.

3.2. Activity of dCK determined by the developed method and a comparative HPLC method

The enzyme dCK is a popular substrate to many pyrimidine and purine analogs such as gemcitabine. Gemcitabine, for which an array of decay assays is available, was selected as
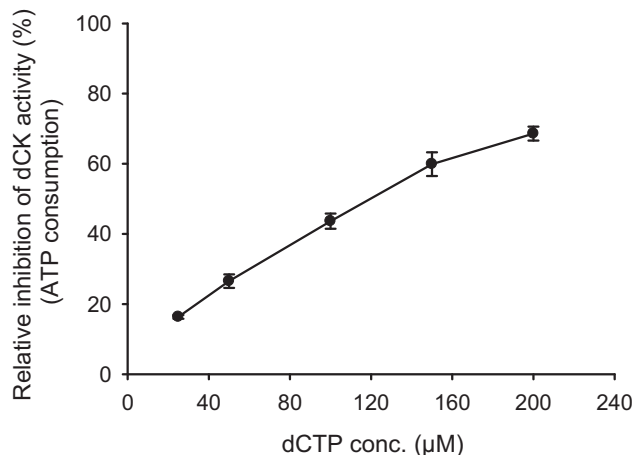

Fig. 4 − **Activity of dCK determined by the developed dCK assay. The dCK activity of HeLa cells was measured by the developed method using 10μM gemcitabine (substrate), 10μM ATP (phosphate donor), and 16.5 μg of the dCK-containing sample from HeLa cells. Measurements were performed in triplicate with the reaction being quenched at 1 minute, 2 minutes, 4 minutes, 6 minutes, 8 minutes, and 10 minutes. ATP = adenosine triphosphate; dCK = deoxycytidine kinase.**

the substrate in the comparative study. The developed luminescence assay was performed in a white 96-well plate with protein extracts from human HeLa cells, and Kinase-Glo reagent, a commercially available cocktail solution that arrests the kinase reaction and develops luminescence signal simultaneously, was added to determine dCK activity. As shown in

Fig. 5 − **Activity of dCK determined by the comparative high-performance liquid chromatography method. Sample and reaction condition were the same as that in Fig. 4. (A) Representative chromatogram of gemcitabine analysis with a United States Pharmacopeia tailing factor of less than 1.5. (B) Measurement of dCK activity based on the kinase reaction quenched at 1 minute, 2 minutes, 4 minutes, and 10 minutes. dCK = deoxycytidine kinase.**

Fig. 3 − **Inhibition of dCK activity by dCTP. Five concentrations of dCTP (25 μg, 50 μg, 100 μg, 150 μg, and 200μM) were used to inhibit dCK activity of HeLa cells in this experiment. ATP = adenosine triphosphate; dCK = deoxycytidine kinase; dCTP = deoxycytidine triphosphate.**
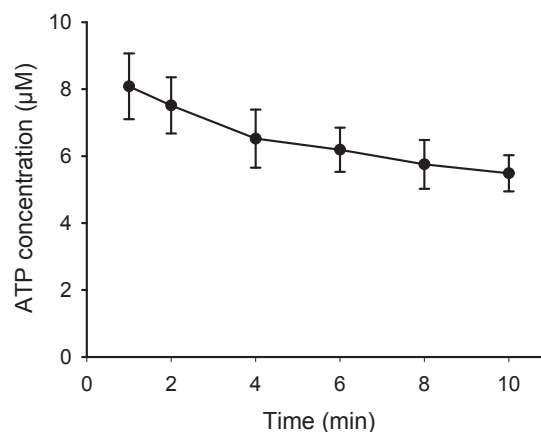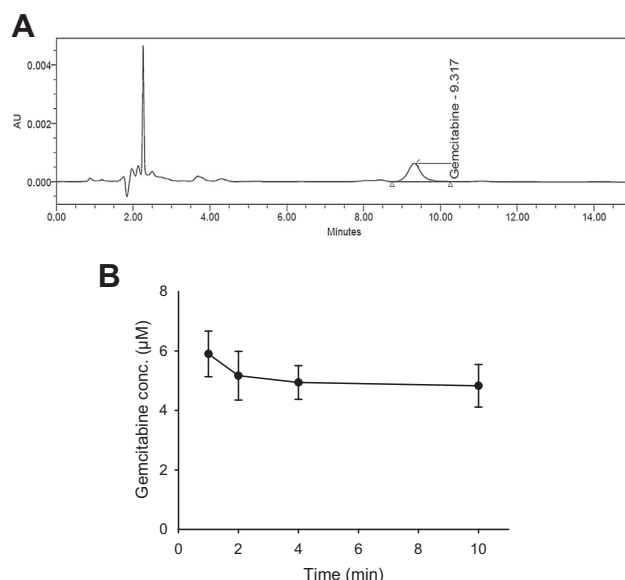
Fig. 4, kinetics of ATP consumption in the reaction milieu was determined quickly using a thermostat microplate reader. The amount of residual ATP decreased gradually over time, indicating that the dCK-mediated phosphate transfer reaction was monitored properly by the developed assay. Alternatively, based on the measurements of substrate decay, the dCK activity of the same HeLa cell sample was also determined comparatively by an HPLC method. Fig. 5 illustrates that as gemcitabine (with a peak at 9.3 minutes) was clearly separated from other reaction components in the chromatogram, dCK activity of the same HeLa cell sample was obtained by plotting the kinetics of gemcitabine decay against time. The gemcitabine decay plot was similar to the ATP consumption plot because dCK mediates phosphorylation of gemcitabine to gemcitabine monophosphate, and not to its di- or triphosphate forms [17].

The highly similar kinetic plots obtained from two methods suggest that both of them monitored the same event. Fig. 6 shows a side-by-side comparison of dCK activity determined by the two methods. In this figure, the dCK activity determined by the developed luminescence method was consistent in two separate reaction periods (0−4 minutes and 0−10 minutes). By contrast, a rapid decay phase resulting in greater activity was observed in the 0−4-minute reaction period for the HPLC method, suggesting that this method is not suitable for detecting a rapid kinase reaction owing to its time-consuming nature. In particular, the HPLC method is relatively insensitive for determining dCK activity with nucleoside analogs, as mentioned previously.

3.3. Profiling dCK activity in cancer cells by the developed method

After the feasibility of the developed method was confirmed, it was employed to profile dCK activity in immortal cancer cells, as dCK is known to determine the efficacy of anticancer
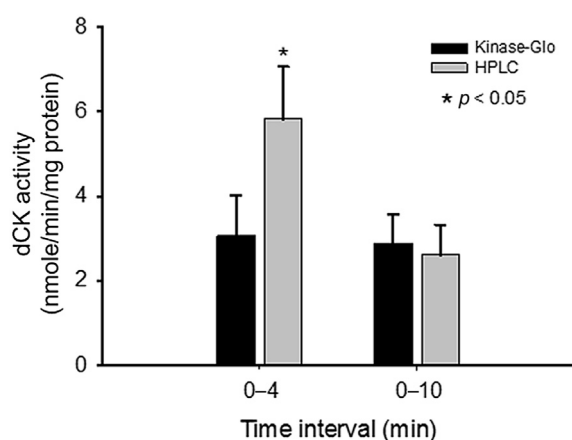

Fig. 6 − Comparison of the developed dCK assay with a comparative HPLC method. Measurement of dCK activity was based on the kinetic data in the periods of 0−4 and 0−10 minutes, whereas dCK activity of HeLa cells performed by these two methods was compared using a *t* test (black bar: the developed dCK assay; gray bar: HPLC method). dCK = deoxycytidine kinase; HPLC = high-performance liquid chromatography.

Table 1 − Activity of dCK in cancer cell lines determined by the developed luminescence assay.

Cell line		dCK activity (nmole/min/mg protein)
Cervix	HeLa	3.06 ± 0.96
Colon	HCT116	2.96 ± 0.52
Lung	LX-1	1.20 ± 0.63
Renal	786-O	2.12 ± 0.07
Breast	MCF-7	2.04 ± 0.40

Data are presented as mean ± SD of three measurements. dCK = deoxycytidine kinase; SD = standard deviation.

nucleoside analogs. In addition to HeLa cells, dCK activity was analyzed in four other cancer cell lines, including the colon (HCT116), lung (LX-1), renal (786-O), and breast (MCF-7) cancer cells, by the developed luminescence assay. Table 1 shows that the dCK activity was 3.06 ± 0.963 nmole/min/mg, 2.96 ± 0.521 nmole/min/mg, 1.20 ± 0.629 nmole/min/mg, 2.12 ± 0.074 nmole/min/mg, and 2.04 ± 0.401 nmole/min/mg protein in HeLa, HCT116, LX-1, 786-O, and MCF-7 cells, respectively.

4. Discussion

Anticancer nucleoside analogs such as gemcitabine are among the most effective drugs used in the clinics. To exhibit therapeutic efficacy, these analogs require cellular uptake followed by phosphorylation to their cytotoxic nucleotide forms. Overexpression and/or dysregulation of protein kinases such as dCK has been proved to affect the severity of disease and efficacy of drug therapy, explaining our interest in developing an efficient and sensitive method to detect dCK activity in biological milieus. The traditional biochemical method used to assay dCK activity in biological matrices is the radioisotope filtration binding assay [4,14], which is always complicated with the handling of isotopes in routine studies.

In this study, we developed a luminescence kinase assay for measuring dCK activity in biological samples such as animal tissues and immortal cancer cell lines. The feasibility of the high-throughput enzymatic assay was demonstrated by the following: (1) its high sensitivity for dCK activity based on corresponding ATP consumption; (2) ideal kinetics of enzymatic reaction including its time and concentration dependence; and (3) high specificities for the substrate (gemcitabine) and inhibitor (dCTP). In addition, suitability of the developed method for biological samples was validated by comparing it with an HPLC method using the same matrix samples.

The major advantages of the developed method over other conventional dCK assays are its efficiency and speed. For the developed luminescence assay, a microplate reader was used to detect the chemiluminescence produced by the couple enzyme reaction, and the throughput time for a measurement was only 0.25 seconds. By contrast, detection time of the comparative HPLC method, as judged by the separation of gemcitabine from milieu components, was generally at least 10 minutes. Overall, speed and complexity of detection of dCK activity in biological samples were improved markedly with the developed facile luminescence method.

Limitations of conventional dCK assays for turbid samples such as blood or tissue debris and their low sensitivity owing to a lack of chromophores for nucleosides were resolved by the developed method. By adopting a simple purification step using anionic exchanger beads to separate dCK from biological contaminants [16], dCK activity in animal tissue can be determined easily, as shown in this study. By contrast, current methods either assay dCK in its pure state or employ filtration and chromatographic procedures to detect dCK in biological matrices. The newly developed method appears to inherit the advantages of chemiluminescence detection techniques, permitting the detection of ATP consumption in blood samples. Furthermore, sensitivity of the developed luminescence method was satisfactory using a minimal amount of sample (protein extract from a well rather than from a flask of cells) for the analysis. We accordingly anticipate a wide application of the developed method to biomarker exploration; further application of this method to improve *in vivo* anticancer drug efficacy is underway in our laboratory.

Acknowledgments

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