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**Running title:** isocratic HPLC for intracellular dFdCTP quantification

## Summary

A deoxycytidine analog, gemcitabine (dFdC), is effective for treating solid tumors and hematologic malignancies. After being transported into cancer cells, dFdC is phosphorylated to dFdC triphosphate (dFdCTP), which is subsequently incorporated into the DNA strand, thereby inhibiting DNA synthesis. Intracellular dFdCTP is the critical determinant for dFdC cytotoxicity, so therapeutic drug monitoring or *in vitro* testing of the capability of cancer cells to accumulate dFdCTP may be informative for optimizing dFdC administration. We have developed a new isocratic-elution HPLC method for quantifying dFdCTP in cancer cells. Samples (500 µl) were eluted isocratically using 0.06 M $Na_2HPO_4$ (pH = 6.9) containing 20% acetonitrile, at a constant flow rate of 0.7 ml/min and at ambient temperature. Separation was performed using an anion-exchange column, TSK gel DEAE-2SW (250 mm x 4.6 mm inside diameter, particle size 5 µl, TOSOH Corp.), and monitored at 254 nm. The standard curve was linear with low within-day and inter-day variability. The lower detection limit (20 pmol) was as sensitive as that of the previous gradient-elution method. dFdCTP was well separated from other nucleoside triphosphates. The method could measure dFdCTP in cultured or primary leukemic cells treated *in vitro*

with dFdC. The method was also applicable to simultaneous determination of dFdCTP and cytarabine triphosphate, the results of which demonstrated the ara-CTP production augmented by the dFdC pretreatment. Thus, our isocratic HPLC assay method will be of great use because of its sensitivity and simplicity as well as its applicability to biological materials.

## Introduction

Gemcitabine (2′,2-difluorodeoxycytidine, dFdC) is the most important deoxycytidine analog to be developed since cytarabine: it has geminal fluorine atoms inserted at the 2′-carbon of the deoxyribofuranosyl ring.[1,2] The drug is active against not only leukemic cells *in vitro* but also several experimental solid tumors such as non-small-cell lung cancer, small-cell lung cancer, and pancreatic cancer.[3-6] Clinically, dFdC has become the standard first-line therapy for patients with advanced pancreatic cancer, and it is rapidly becoming incorporated into first-line regimens in non-small-cell lung cancer and transitional-cell carcinoma of the bladder.[7-9] The efficacy of dFdC has also been evaluated for leukemic patients.[10,11] Thus, dFdC is now used widely for treating both solid tumors and hematologic malignancies.[7-11]

dFdC is a prodrug that requires intracellular activation.[1,2] After being transported into cancer cells, it is phosphorylated via dFdC monophosphate and diphosphate to dFdC triphosphate (dFdCTP), the process of which is catalyzed by several kinases including the rate-limiting enzyme deoxycytidine kinase. dFdCTP is incorporated into DNA at the penultimate position and, after the incorporation of one or more nucleotides, blocks further elongation of the DNA strand. The incorporation

of dFdCTP into DNA is strongly associated with the inhibition of further DNA synthesis, so intracellular dFdCTP concentration is considered to be the most critical determinant of dFdC cytotoxicity.[1,2,12]

As demonstrated in the case of a similar deoxycytidine analog, cytarabine, the critical parameter for predicting the clinical efficacy of a nucleoside analog is not the plasma drug concentration but its triphosphate form in the cancer cells.[13-15] Then, pharmacokinetic evaluation of dFdCTP will provide crucial information necessary for the scheduling and dosing of dFdC. The most widely used method for measuring the intracellular dFdCTP concentration uses gradient-elution ion-exchange HPLC, which was developed by Gandhi et al.[16,17] This is a sensitive method applicable to the measurement of dFdCTP in mononuclear cells and leukemic cells from patients receiving dFdC. However, it requires a complicated computerized system equipped with multiple pumps for regulating two different buffers. Moreover, gradient-elution HPLC methods usually induce base-line drift, which might interfere with the quantification of the small peak in biological materials. To overcome these disadvantages, we have developed an isocratic-elution HPLC method for measuring dFdCTP in cancer cells. In contrast to the gradient-elution method, the isocratic model is simple and inexpensive as it can be run by a single mechanical pump and

induces no base-line drift. We demonstrate that our method is precise, sensitive, and applicable to biological samples.

## Materials and Methods

### Chemicals

$Na_2HPO_4$, acetonitrile (HPLC grade), phosphoric acid (85%), and perchloric acid (60%) were purchased from Nacalai (Kyoto, Japan). dFdC and dFdCTP were kindly provided by Eli Lilly Corp. (Indianapolis, IN, USA). Cytarabine was purchased from Sigma (St. Louis, MO, USA).

### Preparation of standard dFdCTP in aqueous solution

dFdCTP was dissolved in distilled water to make a stock solution (2 mM), from which six different concentrations of dFdCTP solution were made. These standard solutions were stored at -80°C before use.

### HPLC apparatus and chromatographic condition

The HPLC apparatus consisted of a pump (CCPM-II; TOSOH Corp., Tokyo, Japan),

an autosampler (AS-8020; TOSOH Corp.), an in-line degasser (SD-8022; TOSOH Corp.), and a variable-wavelength detector (UV-8020; TOSOH Corp.). The system was controlled and the data were analyzed using a personal computer installed with LC-8020 software (TOSOH Corp.).[14,18] Samples were eluted isocratically with 0.06 M $Na_2HPO_4$ (pH = 6.9) containing 20% acetonitrile, at a constant flow rate of 0.7 ml/min and at ambient temperature. Separation used an anion-exchange column, TSK gel DEAE-2SW (250 mm x 4.6 mm inside diameter, particle size 5 μl, TOSOH Corp.), and was monitored at 254 nm, the maximum UV absorption of dFdCTP.

**Standard curve**

To determine the standard curve for the assay, a 500-μl-aliquot from each diluted aqueous solution of standard dFdCTP ranging between 20 pmol and 2 nmol (20 pmol, 50 pmol, 100 pmol, 200 pmol, 500 pmol 1 nmol, and 2 nmol) was applied to the HPLC. The solutions were measured in triplicate on 3 separate days to determine the within-day and inter-day variation. Data were combined and plotted to determine the correlation between the amount of dFdCTP and the peak area. The standard curve was fitted by the weighted least-squares linear regression analysis method using the equation $y=ax+b$. dFdCTP in unknown samples was quantified by extrapolating the

peak area count into the equation of the standard curve.

**Cultured or primary leukemic cells**

To validate the applicability of the method to biologic materials, cultured or primary leukemic cells were used. Human leukemia HL60 cells were cultured in RPMI 1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma) in 5% $CO_2$ humidified atmosphere at 37°C. Primary leukemic cells were obtained with informed consent from a patient with chronic lymphocytic leukemia. Peripheral blood (10 ml) was drawn into a heparinized tube and the Ficoll-Paque density gradient centrifugation procedure was used to isolate leukemic lymphocytes.[19] The cultured or primary cells, treated or untreated with dFdC, were then washed twice with fresh media, followed by centrifugation (400 *g*, 10 min, 4°C) to collect the cell pellet in a microcentrifuge tube. The cells were added to 200 μl of 0.3 M cold perchloric acid, and the mixture was vortexed for 10 s then allowed to stand for 15 min at 4°C. The acidic supernatant was isolated by centrifugation of the sample (15,600 *g*, 20 s, 4°C), followed by neutralization with 100 μl of 0.5 N potassium hydroxide. After further centrifugation (15,600 *g*, 20 s, 4°C), the neutralized supernatant was obtained as an acid-soluble fraction (ASF), a nucleotide pool.[14,18] The volume of each ASF sample

was adjusted to 700 μl by the addition of water, from which a 500 μl aliquot was applied to the chromatographic analysis. The intracellular concentration of dFdCTP was expressed as pmol/$10^7$ cells. The samples were stored at -80°C until analyzed.

**Preparation of standard dFdCTP mixed with ASF**

To further confirm that the method was applicable to biologic samples, another set of dFdCTP standards was made using an ASF. Various amounts of dFdCTP stock solution were mixed with an ASF extracted from untreated HL60 cells, and the volume of the samples was adjusted to 700 μl by the addition of water. A 500 μl aliquot from each dFdCTP-ASF solution was applied to the HPLC to give final amounts of dFdCTP that were equivalent to the aqueous solutions described above. The precision was then assessed as within-day and inter-day assay variation for the measurement of dFdCTP in the ASF.

**Calculations and statistical analyses**

The standard curve was obtained using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). The mean, coefficient of variation (CV) and standard deviation for the dFdCTP measurements were obtained using Microsoft

Excel software (Microsoft Corporation, Redmond, WA, USA).

## Results

### Standard curve and validation

To determine the standard curve, six standard aqueous solutions of dFdCTP were applied in triplicate to our HPLC scheme on 3 separate days. A linear relation was obtained between the amount of dFdCTP present and the peak area (Fig. 1). The within-day and inter-day variabilities were determined at different amounts of dFdCTP for validation. The variations were low, with all %coefficient of variation (%CV) values < 10% (Table 1). The lower and upper limits of quantification, the lowest and highest amounts of dFdCTP that gave %CV < 10%, were 20 and 2000 pmol, respectively. The low limit was as sensitive as that of the previous HPLC assay method (25 pmol).[16,17] Thus, these results show that our HPLC method was precise and sensitive for quantification of dFdCTP.

### Separation of dFdCTP

To assess the separation of dFdCTP, a mixture of standard nucleotides (CTP, UTP,

ATP, GTP, and dFdCTP) was injected onto the HPLC. The dFdCTP peak was clearly separated from the other nucleotides (Fig. 2A), with a retention time of 93 min. The retention times of standard deoxyribonucleotides (dATP, dCTP, dTTP, dGTP) were almost identical to those of the corresponding ribonucleotides (data not shown), which did not overlap the dFdCTP peak.

To see if the present HPLC condition was applicable to the measurement of dFdCTP in biological samples, nucleotide pools extracted from leukemic cells were applied. Fig. 2B represents a blank chromatogram of an ASF extracted from untreated human leukemia HL60 cells. Endogenous nucleoside triphosphates (CTP, UTP, ATP, and GTP) were clearly separated in the same way (Fig. 2B), as had been demonstrated using the mixture of standard nucleotides (Fig. 2A). The chromatogram did not contain any peaks between ATP and GTP that might otherwise have interfered with the dFdCTP peak. When the ASF was co-eluted with the standard dFdCTP solution, the peak of dFdCTP was clearly independent of the other endogenous peaks of the leukemic nucleotides (Fig. 2C), confirming the applicability of the method to biologic samples.

To further investigate the method's clinical utility, it was applied to the measurement of dFdCTP generated in leukemic cells *in vitro*. An ASF was extracted

from HL60 cells after treatment with dFdC, and then applied to the HPLC analysis. Even following incubation with the lowest drug concentration for the shortest time period, the presence of dFdCTP in the leukemic cells was clear on the chromatogram (Fig. 2D). Thus, these results clearly show that the dFdCTP is separated from the nucleotide pool of the biologic material using our HPLC conditions.

**Validation of the dFdCTP measurement in biological samples**

To validate our method in biologic samples, it was applied to measuring known concentrations of dFdCTP mixed with the ASF extracted from untreated HL60 cells. The dFdCTP-ASF solutions were injected onto the HPLC to give final quantities of dFdCTP equivalent to those tested in aqueous solution. Measurements of these dFdCTP-ASF solutions at four different amounts (200 pmol, 500 pmol, 1000 pmol, 2000 pmol) in triplicate were performed on 3 separate days. The within-day and inter-day variabilities (%CV) were 1.3% and 5.7% for 200 pmol, 1.9% and 0.7% for 500 pmol, 3.6% and 0.4% for 1000 pmol, and 2.2% and 2.2% for 2000 pmol, respectively. Moreover, the peak areas were similar to and well correlated with those generated by the dFdCTP-water solutions (Fig. 3). This indicates that dFdCTP was quite stable in the ASF and that its measurement was not disturbed by the

endogenous ASF peaks. Thus, these results strongly suggest that our HPLC method is applicable to the quantification of dFdCTP in biologic samples.

**dFdCTP generation in leukemic cells *in vitro***

To confirm the applicability of our method to biologic samples, the production of dFdCTP in cultured or primary leukemic cells was determined. When HL60 cells was incubated with dFdC in vitro, the accumulation of dFdCTP was time- and concentration dependent (Fig. 4A&B). However, the accumulation was maximized at 2-5 μM dFdC (Fig. 4B). Similarly, the concentration- and time-dependent accumulation of the intracellular dFdCTP was observed in primary leukemic cells from a patient with chronic lymphocytic leukemia (Fig. 4C). Thus, our HPLC assay method could measure the intracellular dFdCTP concentrations in cancer cells.

**Separation between cytarabine triphosphate and dFdCTP**

One of the strategies for overcoming the chemo-resistance of leukemia is to augment the accumulation of the triphosphate form of the key anti-leukemic drug cytarabine in leukemic cells using other nucleoside analogs such as fludarabine or dFdC.[20,21] When HL60 cells were treated with dFdC combined with cytarabine, our HPLC

method separated cytarabine triphosphate (retention time 66 min) from dFdCTP (Fig. 5A&B). The analysis revealed that the preincubation with dFdC augmented the cytarabine triphosphate production by 1.6 fold (Fig. 5C). Thus, the method is capable of simultaneously measuring the triphosphates of these two compounds.

## Discussion

We have developed a new, simple and sensitive analytical method for quantifying dFdCTP in cancer cells using isocratic HPLC. dFdCTP was clearly separated from adjacent nucleotides (Fig. 1), and the assay variation was low, with %CV < 10% (Table 1). The method is as sensitive as the commonly used gradient-elution method. Its applicability to biologic materials was demonstrated by the measurement of dFdCTP concentrations in cultured or primary leukemic cells (Fig. 2 and 4). Our HPLC scheme is also applicable to simultaneous determination of dFdCTP and cytarabine triphosphate (Fig. 5).

The previously reported HPLC method for determining intracellular dFdCTP concentration used a Partisil-10 SAX anion-exchange column (250 mm x 4 mm inside diameter, Whatman, Inc. Clifton, NJ, USA) and two buffers, buffer A (5 mM $NH_4H_2PO_4$,

pH = 2.8) and buffer B (0.75 M $NH_4H_2PO_4$, pH = 3.49). The separation used a concave gradient elution (curve #9) starting with 65% of buffer A and 35% of buffer B and finishing with 100% buffer B, at a rate of 3 ml/min for 30 min.[16,17] Although this method takes less running time than ours, it requires a computerized system to regulate two pumps. In contrast, our new isocratic HPLC method uses a single buffer and can be controlled not only using a computerized system but also using a conventional, mechanical single pump. Moreover, isocratic elution does not induce base-line drift (Fig. 2), so allows accurate measurement of small amounts of dFdCTP in crude biologic extracts, which lead to the excellent sensitivity of the present method (Fig. 1, Table 1).

Previous reports demonstrated that in peripheral mononuclear cells or leukemic cells dFdCTP accumulation reached a plateau when the plasma dFdC concentration exceeded 15-20 μM.[22-24] Studies with leukemic patients suggested that a dose rate of 10 mg/$m^2$/min would produce plasma dFdC concentrations > 20 μM and maximize the rate of dFdCTP accumulation.[23,25] In contrast, Fig. 4 demonstrated the intracellular dFdCTP production was maximized at 2-5 μM dFdC in media. This might be attributed to the difference of the cell types. The results also suggest that the intracellular pharmacokinetics vary among cancers.

dFdCTP successfully augmented cytarabine trihposphate production in HL60 cells (Fig. 5). Intracellular dFdCTP inhibits ribonucleotide reductase, an enzyme responsible for de novo synthesis of deoxyribonucleotides. The inhibition of ribonucleotide reductase results in a decline in the intracellular dCTP pool, thereby decreasing dCTP-mediated feedback inhibition of deoxycytidine kinase. Enhanced deoxycytidine kinase consequently augments production of cytarabine triphophsate.[3] Therefore, the present HPLC method would also be useful for pharmacologic evaluation of the combination effect between cytarabine and dFdC.

Clinical studies have been conducted to optimize dFdC therapy by adjusting its dose and schedule to obtain the maximum accumulation of dFdCTP in cancer cells, since intracellular dFdCTP is critical to dFdC cytotoxicity.[22-25] Because intracellular pharmacokinetics vary widely between patients and cancers,[15] therapeutic drug monitoring at an intracellular level may be necessary for better individualization. Moreover, after resection of tumors such as pancreatic cancer and lung cancers, *in vitro* testing of the tumor's sensitivity to dFdC or its capability to accumulate dFdCTP might provide crucial information to guide the decisions about adjuvant chemotherapy and regimen selection.[26,27] In either case, the intracellular dFdCTP concentration needs to be determined easily and inexpensively in hospitals

on a daily basis. Thus, for these purposes, our isocratic HPLC method has the great advantages of sensitivity and simplicity as well as applicability to biologic materials.

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## Legends

**Fig. 1.**

Standard curve for dFdCTP measurement. Various concentrations of standard dFdCTP (20, 50, 100, 200, 500, 1,000, and 2,000 pmol ) in aqueous solution were applied in triplicate to our HPLC scheme on 3 separate days. A linear relation was obtained between dFdCTP amount and peak area, using all the data sets. The parameters for the curve were: y-intercept = -12.1 ± 4.7, slope = 0.92 ± 0.01 ($r^2$ = 0.9981, $P < 0.0001$, for the linear regression line).

**Fig. 2.**

HPLC profiles of nucleotides. (A) Standard nucleotides (ATP, CTP, UTP, GTP, and dFdCTP) dissolved in water were applied to our HPLC scheme; (B) an acid-soluble fraction was extracted from untreated leukemic HL60 cells ($2 \times 10^7$ cells), and applied to the HPLC; (C) the same acid-soluble fraction was co-eluted with standard dFdCTP; (D) an acid-soluble fraction was extracted from HL60 cells after 0.5 h incubation with 0.1 μM dFdC and applied to the HPLC.

**Fig. 3.**

Correlation between dFdCTP measurements in aqueous and ASF solutions. Measurements of dFdCTP in water or ASF were performed in triplicate on 3 separate days. The peak area counts were similar and closely correlated (slope = 1.01 ± 0.01, y-intercept = 0, x-intercept = 0, $r^2$ = 0.99, P < 0.0001, for the linear regression line).

**Fig. 4.**

dFdCTP production in leukemic cells. (A) HL60 cells (1 × $10^6$ cells/ml, 10 ml) were incubated with 0.1 μM (o) or 0.5 μM (●) dFdC for the indicated time periods (0, 0.5, 1.0, 1.5 h). Alternatively, (B) the cells were incubated for 1.5 h with the indicated concentrations (0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 μM) of dFdC. (C) Primary leukemic cells (1 × $10^6$ cells/ml, 10 ml) from a patient with chronic lymphocytic leukemia were incubated for 2.0 h (o) or 4.0 h (●) with the indicated concentrations (0, 1.0, 2.0 μM) of dFdC. The samples underwent the extraction of acid soluble fraction, and were subsequently analyzed by our HPLC method to determine the intracellular dFdCTP concentrations.

**Fig. 5.**

Separation of cytarabine triphosphate from dFdCTP. HL60 cells ($1 \times 10^6$ cells/ml, 10 ml) were incubated with (B) or without (A) 10 μM dFdC for 2.0 h, followed by washing, resuspension in fresh medium, and incubation with 10 μM cytarabine for 3 h. The samples were then applied to the HPLC. The ara-CTP production was augmented by the dFdC preincubation (C).

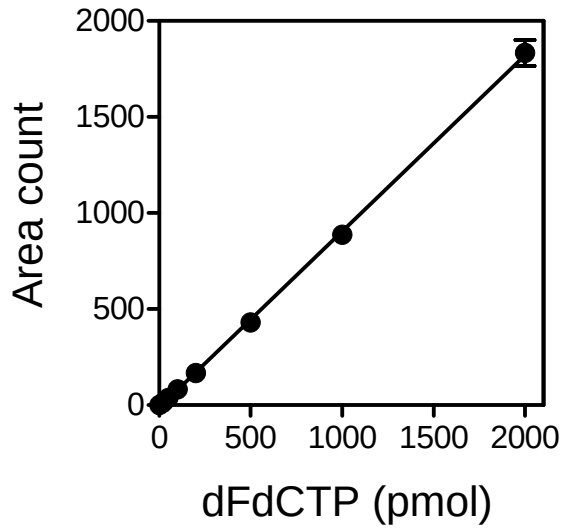
Table 1

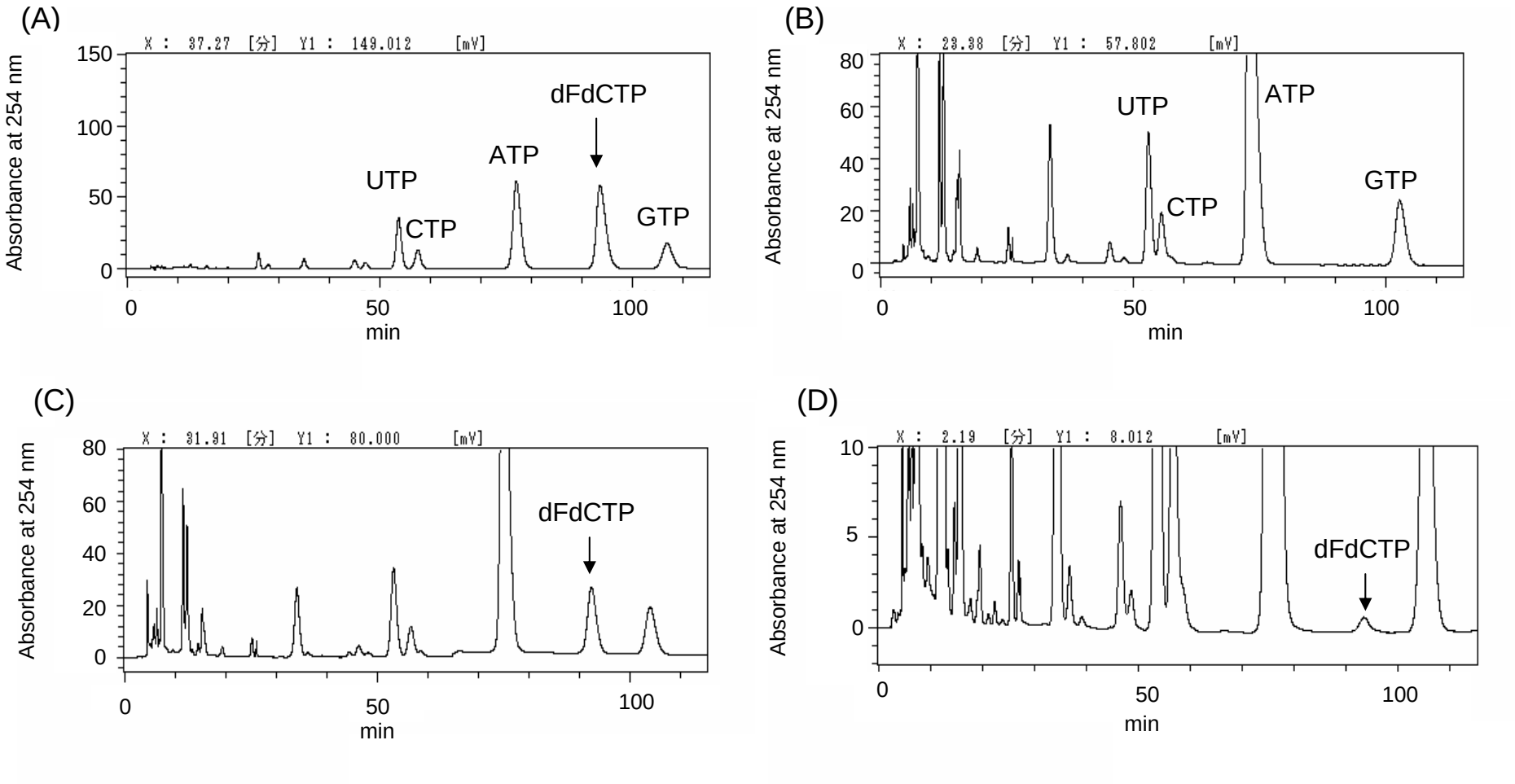
Within-day and inter-day variation for dFdCTP in aqueous solution

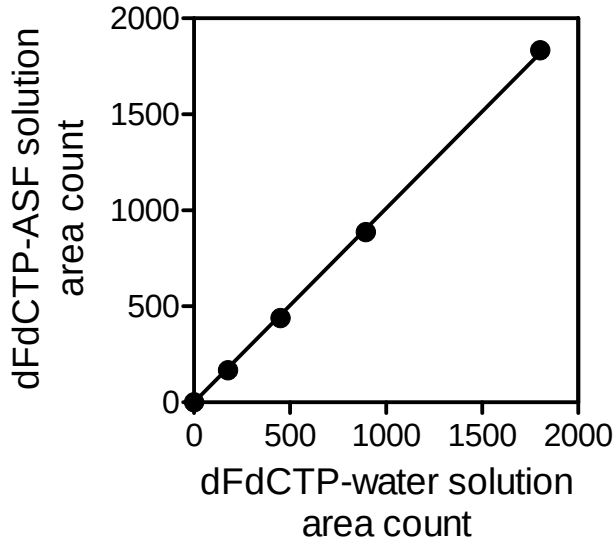
| dFdCTP (pmol) | variation (Mean / SD / %C.V.) | |
|---|---|---|
| | Within-day (day 1) | Inter-day |
| 20 | 11.0 | 13.0 |
| | 0.9 | 0.5 |
| | 8.0 | 3.5 |
| 50 | 39.7 | 34.8 |
| | 1.6 | 0.2 |
| | 4.1 | 0.6 |
| 100 | 84.4 | 81.0 |
| | 5.7 | 4.0 |
| | 6.8 | 4.9 |
| 200 | 167.9 | 166. 5 |
| | 9.6 | 1.7 |
| | 5.7 | 1.0 |
| 500 | 439.8 | 439.4 |
| | 15.9 | 7.2 |
| | 3.6 | 1.6 |
| 1000 | 880.6 | 893.1 |
| | 26.9 | 6.5 |
| | 3.1 | 0.7 |
| 2000 | 1867.0 | 1800.3 |
| | 54.1 | 70.6 |
| | 2.9 | 3.9 |

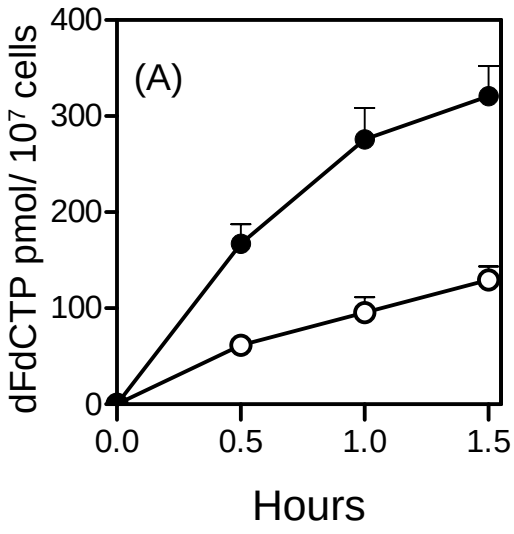
Standard aqueous solutions of dFdCTP were measured in triplicate on 3 separate days. The within-day variation given is that measured on day 1. The inter-day variation was calculated from the mean of each day's peak area count.

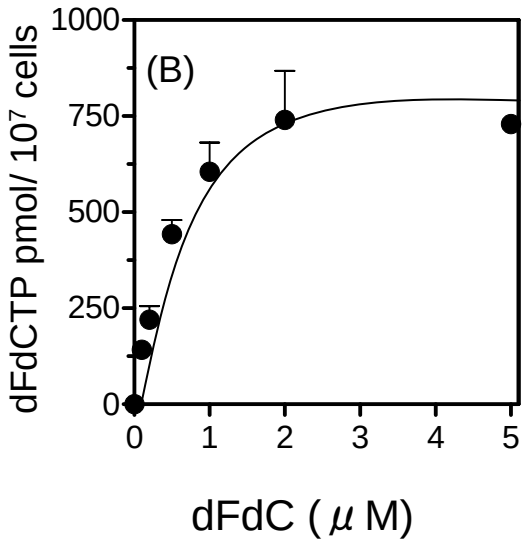
C.V. ; coefficient of variation

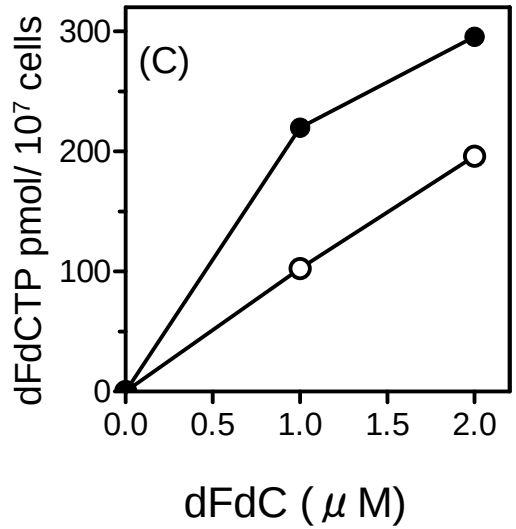

2000
1500
1000
500
0
Area count
0
500
1000
1500
2000
dFdCTP (pmol)


(A)
X : 37.27 [分] Y1 : 149.012 [mV]
Absorbance at 254 nm
150
100
50
0
UTP
CTP
ATP
dFdCTP
GTP
0
50
100
min
(B)
X : 23.38 [分] Y1 : 57.802 [mV]
Absorbance at 254 nm
80
60
40
20
0
UTP
CTP
ATP
GTP
0
50
100
min
(C)
X : 31.91 [分] Y1 : 80.000 [mV]
Absorbance at 254 nm
80
60
40
20
0
dFdCTP
0
50
100
min
(D)
X : 2.19 [分] Y1 : 8.012 [mV]
Absorbance at 254 nm
10
5
0
dFdCTP
0
50
100
min

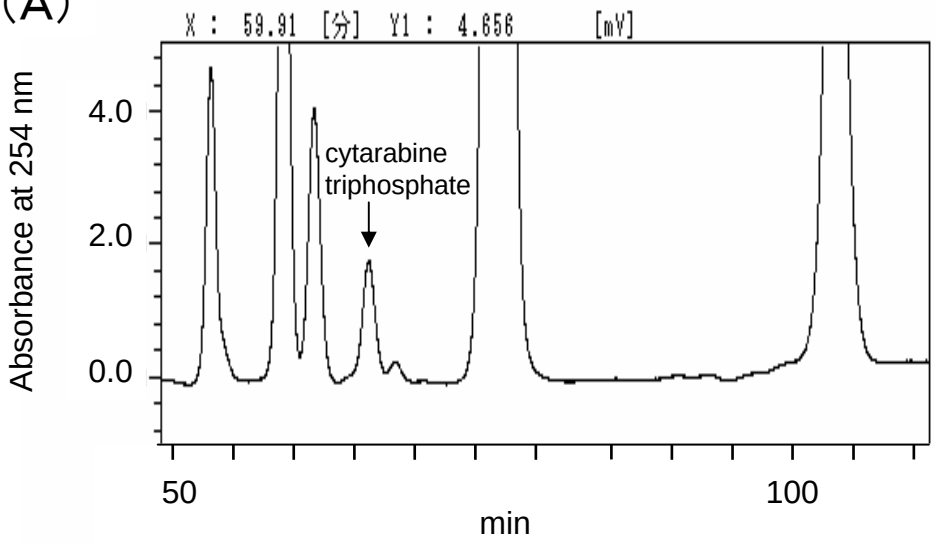

2000
1500
1000
500
0
0
500
1000
1500
2000
dFdCTP-ASF solution
area count
dFdCTP-water solution
area count


(A)
dFdCTP pmol/ 10^7 cells
400
300
200
100
0
0.0
0.5
1.0
1.5
Hours

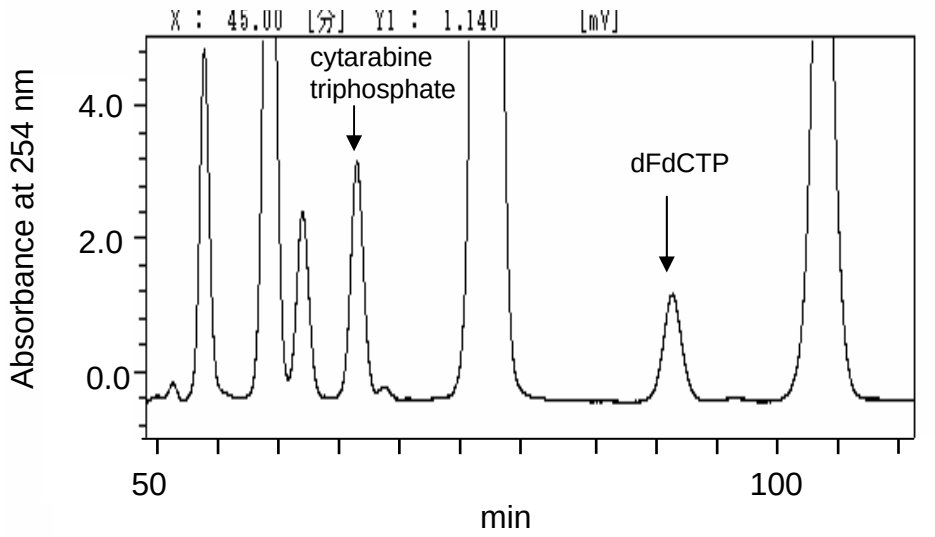

(B)
dFdCTP pmol/ 10^7 cells
1000
750
500
250
0
0
1
2
3
4
5
dFdC ( μ M)


(C)
dFdCTP pmol/ 10^7 cells
300
200
100
0
0.0
0.5
1.0
1.5
2.0
dFdC ( μ M)

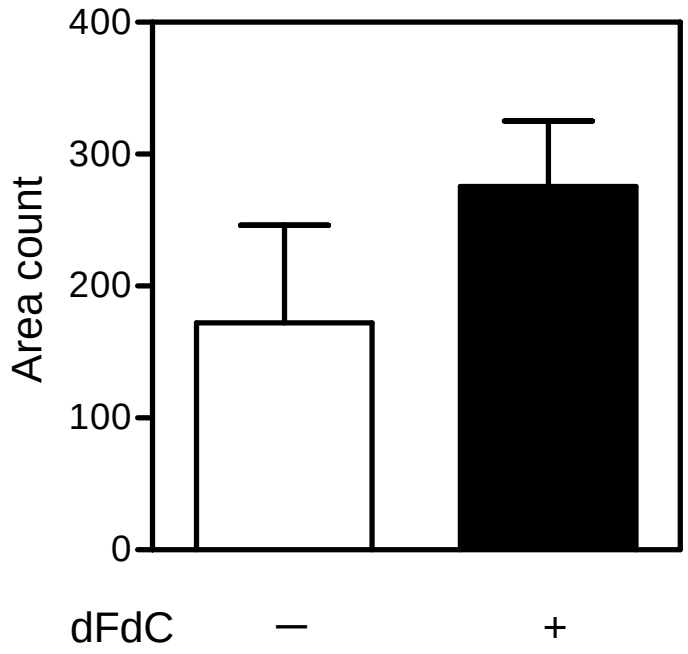
(A)


X : 59.91 [分] Y1 : 4.656 [mV]
Absorbance at 254 nm
4.0
2.0
0.0
cytarabine
triphosphate
50
100
min

(B)


X : 45.00 [分] Y1 : 1.140 [mV]
Absorbance at 254 nm
4.0
2.0
0.0
cytarabine
triphosphate
dFdCTP
50
100
min

(C)


Area count
400
300
200
100
0
dFdC
−
+