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Isocratic ion-exchange chromatographic assay for the nucleotide gemcitabine triphosphate in human white blood cells

Rolf W. Sparidans[a,*], Mirjam Crul[b,c], Jan H.M. Schellens[a,c], Jos H. Beijnen[a,b]

[a]*Division of Drug Toxicology, Department of Biomedical Analysis, Faculty of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands*

[b]*Department of Pharmacy and Pharmacology, Slotervaart Hospital/The Netherlands Cancer Institute, Louwesweg 6, 1066 EC Amsterdam, The Netherlands*

[c]*Department of Medical Oncology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands*

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Abstract

An isocratic bio-analytical assay for the nucleotide gemcitabine triphosphate (2′,2′-difluorodeoxycytidine 5′-triphosphate, dFdCTP) in human white blood cells (leukocytes) has been developed and validated. The method is based on ion-exchange liquid chromatography and ultraviolet detection (275 nm). dFdCTP is isolated from the matrix by extraction with perchloric acid while the sample is chilled on ice. After neutralization with potassium hydroxide and removal of the potassium perchlorate precipitate, with the sample still chilled on ice, the mixture is injected into the chromatograph. The method has been validated in the range 0.4–20 μ*M*, 0.4 μ*M* (~20 pmol/10^6 cells) being the lower limit of quantification, using erythrocytes as a substitute for leukocytes. Precisions and accuracies both meet the current requirements for a bioanalytical assay. The stability of dFdCTP in intact mononuclear blood cells on ice is strongly limited (half-life ~100 min) and after freezing the half-life of the analyte in the cellular lysate is ~30 min. On the other hand, no degradation was observed for dFdCTP for at least ~24 h in perchloric acid extracts on ice or in neutralized extracts at ambient temperature. The applicability of the assay was demonstrated in white blood cells of a patient with advanced non-small cell lung cancer receiving i.v. gemcitabine.

Keywords: Nucleotide; Gemcitabine triphosphate

1. Introduction

Gemcitabine triphosphate (2′,2′-difluorodeoxycytidine 5′-triphosphate, dFdCTP; Fig. 1) is the most important active metabolite of gemcitabine, a cytotoxic deoxycytidine analogue. This compound is intracellularly formed by phosphorylation by deoxycytidine kinase and can be found in white blood cells (WBC, leukocytes, mononuclear blood cells) containing this enzyme. In addition to intracellular activation, gemcitabine can be extracellularly deactivated to 2′,2′-difluoro-2′-deoxyuridine by deoxycytidine deaminase. The mechanism of action of dFdCTP is presumably incorporation into DNA, leading to inhibition of DNA synthesis and ultimately cell death. In addition, dFdCTP may interfere with

*Corresponding author. Tel.: +31-30-253-7376; fax: +31-30-253-5180.

E-mail address: r.w.sparidans@pharm.uu.nl (R.W. Sparidans).

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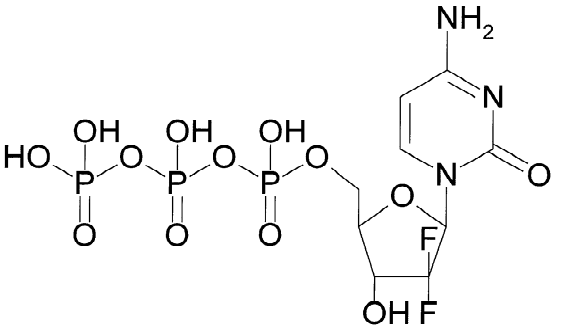

Fig. 1. Chemical structure of dFdCTP.

the ribonucleotide metabolism and can also be incorporated in RNA [1]. Gemcitabine diphosphate (dFdCDP), the precursor of dFdCTP, is an inhibitor of ribonucleotide reductase. This inhibition reduces the deoxynucleotide pools, necessary for DNA synthesis. Thus, dFdCTP can potentate the action of dFdCTP.

In preclinical models, a good correlation between dFdCTP incorporated in DNA and cytotoxic activity of gemcitabine was demonstrated [2] and therefore it is generally believed that dFdCTP is the pharmacologically most important metabolite of gemcitabine. Consequentially, pharmacokinetic and clinical studies are preferably monitored by measuring dFdCTP levels in white blood cells in addition to the parent drug in plasma [3–6].

Investigations into chromatographic assays for dFdCTP finally all lead back to the work of Rose and Brockman [7]. They were the first to use gradient ion-exchange high-performance liquid chromatography (HPLC) as a tool for the intracellular determination of a triphosphate metabolite of a nucleoside analogue drug, 9-β-D-arabinofuranolyladenine 5′-triphosphate. This assay was later adapted, modified and used in preclinical and clinical studies of gemcitabine, first by Plunkett et al. [3–5,8,9] and later by Peters et al. [1,6,10–13]. Both research groups use gradient ion-exchange HPLC with ultraviolet (UV) detection for the analysis of dFdCTP, sometimes in combination with other nucleotides, in various cellular matrices after an acid extraction with perchloric acid [9] or trichloroacetic acid [1]. However, a validated assay has not yet been published including the stability of the triphosphate anabolite under experimental conditions. We now present a validated isocratic assay for dFdCTP in human white blood cells.

2. Experimental

2.1. Chemicals

Gemcitabine triphosphate disodium, present in a mixture with its diphosphate analogue, was kindly donated by Eli Lilly (Indianapolis, IN, USA). Acetonitrile (gradient grade) was provided by Biosolve (Valkenswaard, The Netherlands) and phosphoric acid (85%, w/w) by Merck (Darmstadt, Germany). Perchloric acid (70% (w/v), p.a.) was obtained from Acros Chemicals (Geel, Belgium). Water was home-purified by reversed osmosis on a multi-laboratory scale and all other chemicals were of analytical grade from Sigma (St. Louis, MO, USA).

Blank, drug-free human red blood cells (RBC, erythrocytes) were obtained from the Bloedbank Midden Nederland (Utrecht, The Netherlands). These cells were diluted 1/20 (v/v) with phosphate-buffered saline (PBS, Sigma, St. Louis, MO, USA) to obtain 5% (v/v) RBC. This dilution was used as a, sufficiently available, substitute for WBC, containing approximately an equal amount of cellular material compared to the WBC samples. WBC originated from patients with advanced non-small cell lung cancer participating in a phase I clinical trial of gemcitabine in combination with cisplatin [14] at the Netherlands Cancer Institute (Amsterdam, The Netherlands), who all gave their informed consent.

2.2. Equipment

Chromatographic analyses were performed on the following configuration: a model 616 pump with column thermostat (Waters Chromatography, Milford, MA, USA), a Waters in-line degasser, a Waters 717plus autosampler and a Spectroflow 783 variable wavelength detector (Kratos Analytical, Ramsey, NJ, USA). Data were recorded on a model 300GL personal computer (IBM, New York, USA), equipped with a Class-VP chromatography data system (version 4.2, Shimadzu, Kyoto, Japan). A model 5417R centrifuge (Eppendorf, Hamburg, Germany) was also used.

2.3. Chromatographic conditions

Injections (150-μl) were made on a Partisil 10 SAX column (250×4.6 mm, d_p=10 μm, Whatman, Clifton, NJ, USA). The column temperature was maintained at 40 °C. The eluent comprised 25% (v/v) acetonitrile and 75% (v/v) of a 412.5-m*M* ammonium phosphate buffer. The buffer is prepared by titrating 0.75 *M* phosphoric acid to pH 2.6 with 0.75 *M* di-ammonium hydrogen phosphate. This buffer was diluted with water to obtain the eluent buffer. The eluent flow-rate was 2 ml/min and the absorbance wavelength was 275 nm, using a 5-s response time.

2.4. White blood cell sampling and treatment

Patient whole blood samples of 15 ml in heparin tubes were centrifuged at 4 °C and ~1500 *g* for 5 min, the buffy coat at the erytrocyte–plasma interface was then collected and diluted with PBS to 6 ml. This suspension was layered over 4 ml of Ficoll-Hypaque (specific gravity 1.077 g/ml, Pharmacia, Stockholm, Sweden). After centrifugation at ~550 *g* and 4 °C for 20 min (without brake), the interface containing the WBC was collected and washed with 30 ml cold PBS (4 °C). Finally, after centrifugation for 5 min at 4 °C and ~1500 *g*, the cell pellet was suspended in 100 μl PBS and immediately frozen in a dry-ice–ethanol bath before storage at −80 °C and analysis. Samples (10 μl) were used for a Bradford protein assay [15].

2.5. Sample preparation

First 100 μl of a sample are pipetted into a 0.5-ml polypropylene reaction tube, kept on ice and already containing 100 μl of 0.8 *M* perchloric acid. After vortex-mixing and centrifugation at 4 °C and ~10 000 *g* for 1 min, 180 μl of the supernatant are pipetted into a new reaction tube, kept on ice. Next, 40 μl of 2 *M* potassium hydroxide are added and the tube is vortex-mixed. After centrifugation at 4 °C and ~10 000 *g* for 1 min, the supernatant is transferred from the chilled tube to an injection vial with a 250-μl glass insert.

2.6. Validation

Aqueous stock solutions of 24.7 and 15.9 μg/ml dFdCTP, a mixture with its diphosphate analogue, were prepared (with separate weighting) and were stored at −20 °C. For calibration, dilutions in 5% (v/v) erythrocytes were made from the 24.7-μg/ml stock solution to yield 0.38, 0.95, 1.9, 3.8, 9.5, and 19 μ*M* dFdCTP, diphosphate corrected, calibration samples in blood cells, respectively. Least-squares regression, weighted by X^{-2} (reversed squared concentration) was employed, using the height of the dFdCTP peaks.

From the 15.9-μg/ml stock solution, validation (quality control, QC) samples in 5% (v/v) erythrocytes were made at 0.38, 0.96, 3.8 and 12 μ*M*, diphosphate corrected, respectively, and stored at −80 °C. Precisions and accuracies were determined by six-fold analysis on 3 separate days.

For the determination of the extraction yield, dFdCTP samples in perchloric acid extract of the 5% (v/v) red blood cells were prepared at 0.48, 1.92 and 6.0 μ*M* from the same stock as the validation samples. These samples were processed three-fold on 3 different days starting with sampling 180-μl portions. The yield was calculated as the ratio of the response of the validation samples and their corresponding samples in perchloric acid extract.

Individual samples of drug-free WBC are used to test the selectivity of the assay, and six independent samples were spiked to obtain 0.38 μ*M* dFdCTP (LLQ).

The QC samples were also used for stability studies. In addition, pooled WBC samples spiked with dFdCTP were prepared at 4.2 and 4.8 μ*M*. Kinetic experiments were performed for dFdCTP in both WBC (4.2 μ*M*, 0 °C, 0–53 min) and RBC (3.8 μ*M*, 37 °C, 0–68 min) by sampling at different intervals followed by extraction with perchloric acid and further analysis. Perchloric acid extracts of both WBC (4.8 μ*M* dFdCTP) and RBC (3.8 μ*M* dFdCTP) were subjected to both 0 °C and ambient temperature for 18.5 h. Furthermore, perchloric acid extracts, containing both 1.0 and 12.0 μ*M* dFdCTP, of RBC were stored at −80 °C and subjected to three extra freeze–thaw cycles; identical samples were stored at −80 °C for ~7 months. The stability of dFdCTP was also tested at 1 and 12 μ*M* in the

neutralized extracts at ambient temperature in the autosampler for 26 h. Finally, the stability of dFdCTP was investigated in intact mononuclear cells by dividing clinical samples in two portions, the second portion being kept on ice (0 °C, 25–60 min, $n=7$) before freezing and further storage. Both splitted samples were analysed later in the same run.

Samples were collected from a patient receiving 2880 mg (1500 mg/m^2) gemcitabine during a 30-min i.v. infusion. Cell counting was performed using the Bradford protein assay [15] after calibration with samples from healthy volunteers and the determination of the number of cells in these samples with a Casy1 cell counter equipped with an Analyser System Model TT (Sharfe System, Reutlingen, Germany).

3. Results and discussion

3.1. Chromatography

Nucleotides are very hydrophilic and strong ionic compounds. Therefore, the acid extraction of cellular material with subsequent ion-exchange liquid chromatography and UV detection has been a powerful combination for the analysis of these compounds for several decades [16]. Alternatively, ion-pair liquid chromatography [17] and capillary electrophoresis [18] are used nowadays as analytical techniques for these compounds. However, for dFdCTP ion-exchange HPLC is still is the separation technique of first choice [1,9]. Before validating such an assay we first investigated retention and resolution of dFdCTP in combination with the prominent endogenous nucleotides ATP, CTP, GTP and UTP using gradient elution. Ionic strength, pH and the amount of acetonitrile in the eluent were shown to be important parameters. The amount of acetonitrile in the eluent proved to be a useful tool for the optimization of the selectivity, probably by modifying non-ionic interactions with the sorbent. Fig. 2 shows that maximal resolution of dFdCTP from the other nucleotides was obtained at 30% (v/v) acetonitrile. After conversion to an isocratic system this was adjusted to 25% (v/v).

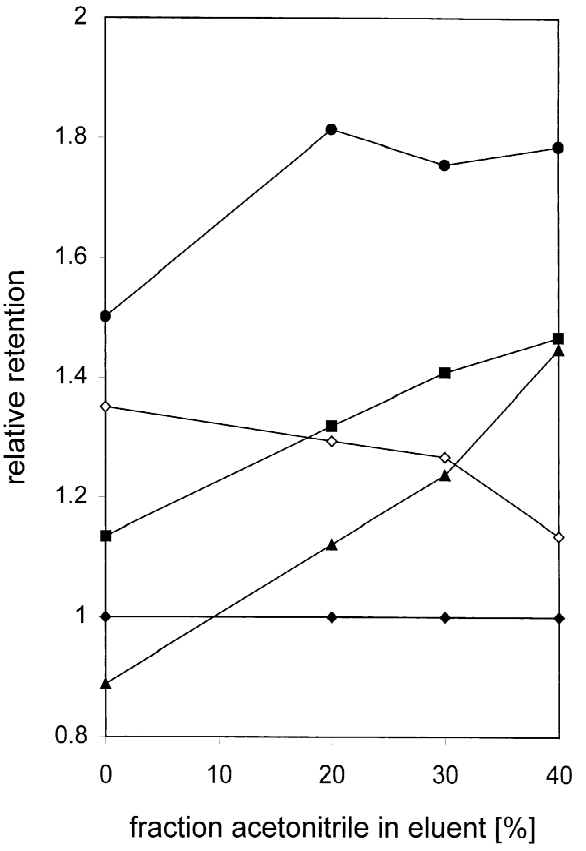

Fig. 2. Retention time of triphosphate nucleotides, relative to dFdCTP, as a function of the amount of acetonitrile (X) in the eluent. (♦) dFdCTP; (■) ATP; (▲) CTP; (●) GTP; (◊) UTP. Column: Partisil 10 SAX column (250×4.6 mm, d_p=10 μm, Whatman), eluent A: (100−X)% (v/v) water and X% (v/v) acetonitrile, eluent B: (100−X)% (v/v) of 0.75 M phosphate buffer (pH 2.6) and X% (v/v) acetonitrile. Gradient: concave (gradient 6) from 35 to 80% B during 45 min. Flow: 3 ml/min. Temperature: 40 °C. Wavelength range: 265–285 nm. Equipment: system controller SCL-10Avp, two LC-10ATvp pumps, SUS mixer, autoinjector SIL-10APvp, diode array detector SPD-M10Avp, Class-VP chromatographic data system, version 5.03 (all Shimadzu, Kyoto, Japan), a personal computer (Hermac Power Systems, Scherpenzeel, The Netherlands) and a temperature control system (Waters Chromatography).

3.2. Sample preparation

Acid soluble nucleotides can be easily and efficiently isolated from biological matrices by extrac-

tion with perchloric acid or trichloroacetic acid. Subsequently, the extracts can be neutralized and the excess of perchlorate or trichloroacetate can be removed using extraction with an alanine–freon mixture [16] or by precipitation of potassium perchlorate after adding potassium hydroxide or carbonate. For dFdCTP, trichloroacetic acid with extraction of perchlorate [1] and perchloric acid with precipitation of perchlorate after the addition of potassium hydroxide [8] have both successfully been used previously. Because of the environmental drawbacks of using freon we choose the second option.

3.3. Validation

Previous to the validation of the assay, the concentration of dFdCTP in both stock solutions was corrected for the relative amount of gemcitabine diphosphate present. Both solutions were injected in duplicate in the chromatograph, resulting in a dFdCTP content of 83.8 and 82.6 mol.% for the 24.7- and 15.9-μg/ml stock solutions, respectively, using the ratios of the tri- and diphosphate peaks at 11.8 and 3.0 min in the chromatogram. The molar extinction was presumed to be equal for both gemcitabine phosphates (this has been reported previously [16] for endogenous nucleotides). The molar concentrations of dFdCTP in the stock solutions were then calculated at 37.9 and 24.0 μ*M*, respectively.

Table 1
Results of back calculated dFdCTP calibration samples ($n=9$)

c, μ*M* (nominal)	Found, μ*M*	RSD, %	Accuracy, %
0.379	0.381±0.027	7.0	100.5
0.948	0.934±0.052	5.6	98.6
1.90	1.89±0.04	1.9	99.9
3.79	3.86±0.12	3.1	101.9
9.48	9.48±0.33	3.5	100.1
19.0	18.9±1.5	2.5	100.0

Examples of chromatograms at different concentrations of dFdCTP in RBC are shown in Fig. 3. The assay was linear over a concentration range from 0.4 to 19 μ*M* in 5% (v/v) RBC, as shown in Table 1. For nine calibration curves the calibration concentrations were back-calculated. The deviation from the nominal concentration for all concentrations was equal to or less than 2% (Table 1) without any sign of non-linearity. The weighted linear regression parameters for these curves (±SD, $n=9$) were: intercept=7±20 μAU; slope=245±19 AU·l·mol^{-1}; standard error=10±4 μAU.

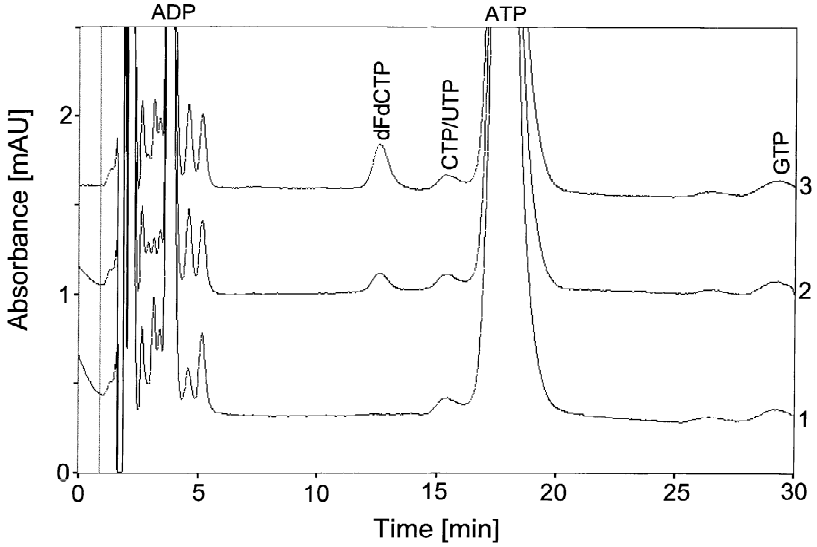

Fig. 3. Chromatograms of dFdCTP in 5% (v/v) RBC. (1) Blank; (2) 0.4 μ*M* spiked to blank RBC; (3) 1 μ*M* spiked to blank RBC.

Table 2
Overall results of validation samples ($n=18$) in 5% (v/v) RBC

c, μM	Repeatability, %	Reproducibility, %	Accuracy, %
12.0	1.2	1.6	102
3.84	2.5	3.1	103
0.960	5.3	5.2	106
0.384	9.3	9.7	110

The results of the validation samples, precision and accuracy at each concentration in the different analytical runs, are listed in Table 2. The lowest level, 0.4 μM, proves to be the LLQ. All values of the precision and accuracy meet the demands for a bioanalytical assay: $\leq$20% for the LLQ and $\leq$15% at higher concentration levels [19,20].

The extraction yield of dFdCTP from 5% (v/v) human erythrocytes was 99, 96 and 98% at 1.0, 3.8 and 12.0 μM, respectively.

In individual blank WBC samples two minor peaks can be observed eluting just before and after dFdCTP (Fig. 4). The quantification of gemcitabine, however, was not disturbed by these endogenous compounds at the LLQ level because all results remain within 20% of the nominal value ($n=6$).

The half-life of dFdCTP was 34 min in lysed WBC at 0 °C and 102 min in 5% (v/v) lysed RBC at 37 °C. In intact WBC, before freezing, the half-life at 0 °C was ~104 min. The poor stability of dFdCTP in WBC samples is probably caused by phosphatase activity of the cellular proteins, even at this low temperature. The stability under other conditions is reported in Table 3.

The extremely poor stability of dFdCTP in lysed WBCs urged us to investigate the possibility of storing the samples as perchloric acid extracts, the matrix after the next step of the sample pretreatment. The stability of dFdCTP in this alternative medium was much better (Table 3). As a result, the samples should now be extracted as soon as possible after collection and purification (centrifugation and Ficoll-Hypaque treatment) previous to storage at −80 °C. During this pre-freezing treatment, cooling of the sample is essential. The order of extraction and storage has not always been reported in previous studies. However, both extraction before storage [13]

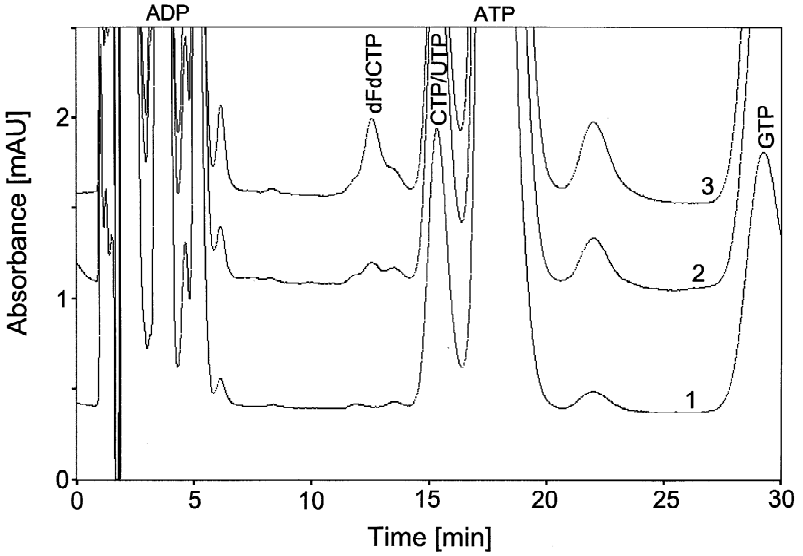

Fig. 4. Chromatograms of dFdCTP in WBC. (1) Blank; (2) 0.4 μM spiked to an individual blank WBC sample; (3) a patient WBC sample containing 1.61 μM dFdCTP.

Table 3
Stability of dFdCTP under different conditions (recovery±SD (%), $n=3$)

Conditions	1.0 μ*M* in RBC	3.8 μ*M* in RBC	12.0 μ*M* in RBC	4.8 μ*M* in WBC
18.5 h at 0 °C in perchloric acid extract		97.9±2.9		98.8±0.6
18.5 h at ambient temperature in perchloric acid extract		64.6±0.3		65.5±2.9
26 h at ambient temperature in neutralized extract	101.0±7.4		101.1±3.0	
11 Days at −80 °C including three extra freeze–thaw cycles	107.3±3.5		106.7±2.9	
7 Months at −80 °C in perchloric acid extract	110.8±2.3		113.5±2.3	

and extraction after storage [4,5] have been used in the past.

A pharmacokinetic curve is shown in Fig. 5, illustrating the applicability of the assay. For patients with advanced inoperable non-small cell lung cancer, gemcitabine with cisplatin is a standard combination for i.v. treatment.

4. Conclusions

dFdCTP can be quantified in WBC using the reported isocratic liquid chromatographic assay to support clinical studies of gemcitabine. Gradient elution, used in all previous studies, is not required. The WBC samples should preferably be stored as their perchloric acid extracts and not as the original biological samples.

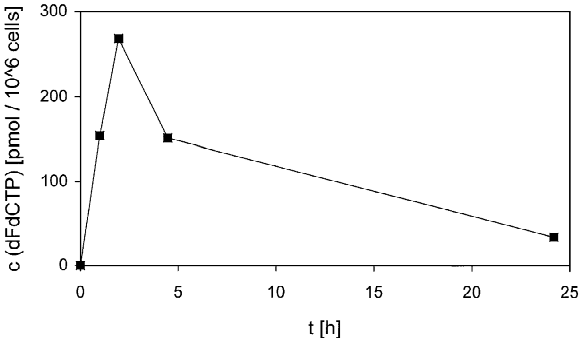

Fig. 5. Pharmacokinetic curve of dFdCTP in WBC from a patient receiving 2880 mg gemcitabine as a 30-min i.v. infusion.

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