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Semi-automated radioassay for determination of dihydropyrimidine dehydrogenase (DPD) activity Screening cancer patients for DPD deficiency, a condition associated with 5-fluorouracil toxicity

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Abstract

Dihydropyrimidine dehydrogenase (DPD) catalyzes the reduction of the naturally occurring pyrimidines, uracil and thymine, and the fluoropyrimidine anticancer drug, 5-fluorouracil (FUra) to 5,6-dihydropyrimidines. Previous studies have demonstrated that cancer patients who are DPD deficient exhibit severe toxicity (including death) following treatment with FUra. To date, the direct measurement of DPD enzyme activity has been the only reliable method to identify DPD deficient cancer patients. We now report a semi-automated radioassay for measuring DPD activity in human peripheral lymphocytes. Following incubation of lymphocyte cytosol (at a fixed protein concentration of 200 μg) with [6-^{14}C]FUra at timepoints ranging from 0 to 30 min, samples are ethanol precipitated, filtered and analyzed by HPLC. Determination of radioactivity is accomplished using an in-line flow scintillation analyzer with automatic quantitation of peaks. This method provides the first specific assay for DPD enzyme activity which is rapid, reproducible and sensitive enough to be used in the routine screening of cancer patients for DPD deficiency prior to treatment with FUra. © 1997 Elsevier Science B.V.

Keywords: Dihydropyrimidine dehydrogenase; Enzymes; 5-Fluorouracil

1. Introduction

Dihydropyrimidine dehydrogenase (EC 1.3.1.2, dihydrouracil dehydrogenase, dihydrothymine dehydrogenase, DPD), is the initial and rate-limiting enzyme involved in the reduction of both uracil and thymine to dihydrouracil and dihydrothymine, respectively [1,2]. DPD catalyzes the first reaction in the three-step catabolic pathway leading to the formation of β-alanine [3]. Clinical interest in DPD activity originated with studies which demonstrated that this enzyme catabolizes more than 85% of an administered dose of the antineoplastic agent 5-fluorouracil (FUra) to dihydrofluorouracil [4]. Recent studies have shown that DPD regulates the amount of FUra available for metabolism, thereby affecting its pharmacokinetics, toxicity and efficacy [5,6].

Deficiency in DPD activity was shown to be responsible for severe FUra toxicity during cancer chemotherapy. Administration of standard doses of FUra to DPD deficient patients resulted in mucositis, granulocytopenia, neuropathy and even death [7–9]. Since the initial reports several years ago, there have been an increasing number of cases reported sug-

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gesting that this disorder may be more frequent [3% based on population studies of DPD activity from peripheral blood mononuclear (PBM) cells] than initially thought [10]. The frequent use of FUra in cancer chemotherapy, the critical role of DPD in FUra pharmacokinetics and the clinical significance of DPD deficiency suggests the potential value of determining DPD activity in cancer patients prior to FUra treatment.

In the present study we describe a new HPLC based, semi-automated radioassay for measuring DPD activity in human peripheral lymphocytes. This assay utilizes [6-^{14}C]FUra; peak detection using an in-line flow scintillation analyzer[1]; and computer software allowing automatic quantitation of peaks. The system allows for continuous multiple sampling with automatic integration and quantitation of dihydrofluorouracil. All chromatograms are stored in data archives following GLP procedures.

To determine whether this assay was useful in the clinical diagnosis of DPD deficiency, samples from recently diagnosed cancer patients were obtained and assayed for DPD activity. These data resulted in the identification of two profoundly DPD deficient cancer patients. These patients are unique in that they were diagnosed as DPD deficient prior to treatment with FUra thus avoiding the life threatening toxicity associated with this syndrome. We conclude that this assay is rapid and simple enough for use in the detection and or screening of DPD deficient patients prior to treatment with FUra.

2. Experimental

2.1. Chemicals

Histopaque, bovine serum albumin, and NADPH were purchased from Sigma (St. Louis, MO, USA). [6-^{14}C]FUra (56 mCi/mmol) was obtained from New England Nuclear (Boston, MA, USA). The purity of the unlabelled and labelled FUra was confirmed by HPLC to be greater than 99%. All other solvents and reagents were purchased in the highest grade available. The primary buffer (buffer A) contained 35 m*M* potassium phosphate (pH 7.4), 2.5 m*M* magnesium chloride and 10 m*M* 2-mercaptoethanol. Since NADPH, the cofactor in the enzyme reaction, is light sensitive and unstable with long term storage, it was freshly prepared.

[1]Additional information (limits of detection, cost, etc.) may be obtained on the internet at http://www.packardinst.com/fsa.htm

2.2. Patients and controls

One hundred healthy volunteer (age range 19–58 years) samples were collected from individuals who had never been treated with FUra. This normal population consisted of faculty, staff and students from this University. Samples from eighty recently diagnosed cancer patients were obtained during initial oncology office visits, prior to any chemotherapy treatment. Informed consent was obtained from both healthy volunteers and cancer patients using an institutionally approved protocol.

2.3. Isolation of mononuclear cells

Since DPD activity is known to follow a circadian pattern of enzyme activity, all blood samples were obtained between 8 a.m. and 9 a.m.. Blood samples (30–60 ml) were drawn from a peripheral vein into a 60 cc syringe containing 5 ml of heparin (1000 units/ml). The syringe was inverted to mix heparin and blood after collection. The blood sample was then layered (in 30 ml aliquots) onto 15 ml of warm (37°C) Histopaque. The blood was fractioned by centrifugation at 2500 *g* for 30 min at 25°C, and the peripheral blood mononuclear (PBM) cells (located between the plasma fraction and Histopaque) were carefully transferred to a clean 50 cc conical centrifuge tube. Residual Histopaque was removed by washing the PBM cells with 25 ml cold (4°C) phosphate-buffered saline (PBS) followed by centrifugation at 1200 *g* for 10 min at 4°C for a total of three times.

2.4. Preparation of cytosol

The fresh PBM cells were resuspended in 300 μl of cold (4°C) buffer A and disrupted (four times for 10 s with a 30 s interval between sonication) on a VirSonic 50 sonicator at 50% power. Following centrifugation at 14 000 *g* for 30 min at 4°C, the supernatant (PBM cytosol) was removed for use in

the subsequent enzyme assays. The protein concentration of the cytosol was determined using the method outlined by Bradford [11] using bovine serum albumin as the standard.

2.5. DPD assay

The standard assay mixture contained 200 μ*M* NADPH, 8.23 μ*M* [6-^{14}C]FUra (56 mCi/mmol), buffer A and 200 μg cytosolic protein in a final volume of 1 ml. Incubations were performed at 37°C. At 5 min time points (from 0 to 30 min), 130 μl of the reaction sample was transferred to a clean eppendorf tube and the reaction was stopped by adding an equal volume of ice cold 100% ethanol. This mixture was then vortexed and placed in a −70°C freezer for at least 10 min to facilitate protein precipitation. The samples were centrifuged for 10 min at 14 000 *g* and filtered through a 0.2 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI, USA) prior to HPLC analysis.

2.6. HPLC analysis

A high performance liquid chromatograph (Hewlett–Packard 1050) was equipped with an automatic injector and an on-line radioisotope flow detector (Radiomatic FLO-ONE Beta, Packard Instrument, Meriden, CT, USA). This system utilizes a Dell XPS P90 computer running Flo-one software which allows continuous multiple sampling with integration and quantitation of peaks of radioactivity. All analyses were performed on 50 μl of sample using two reversed phase columns (25×0.46 cm) connected in series and packed with Hypersil ODS (Jones Chromatography, Lakewood, CO, USA)[2]. Elution was carried out isocratically at a flow rate of 1 ml/min with a mobile phase consisting of 1.5 m*M* potassium phosphate–5.0 m*M* tetrabutylammonium hydrogen sulfate (pH 8.5). Eluent from the columns was directed via a low dead volume connection into the on-line radioisotope flow detector for quantitation of catabolites. A non-gelling scintillation fluid (Ultima-Flo AP, Packard., Downers Grove, IL, USA) was used for all analyses at an effluent to scintillation fluid ratio of 1:3 (v/v).

[2]Attempts in our laboratory to reduce cost and analysis time by using a single HPLC column proved unsuccessful. While it is possible to separate FUra and FUH_2 under these conditions, the peaks become broader with a single column and the assay becomes significantly less sensitive.

2.7. Calculation of DPD activity

Integration and quantitation of peaks of radioactivity was calculated using the Flo-one software provided with the on-line radioisotope flow detector. A quench correction was made using a special quench curve correction program incorporated into the software of this instrument. The quench of a standard radiolabelled sample (while in the mobile phase used during the HPLC analysis) is determined by the channels ratios method [12]. The channels ratios vs. the counting efficiency is stored by the system software. When an actual sample is analyzed with the same mobile phase, the dpm is automatically corrected for quenching. Since there is no dilution in vitro by endogenous metabolites, the nmol of the dihydrofluorouracil can be calculated directly from the peak area.

To determine the specific activity of patient samples, nmol of dihydrofluorouracil formed (y) were plotted against time (x). The slope of the graph (products formed/min) was then calculated by linear regression analysis. The slope was then divided by the amount of protein added to obtain the final result (DPD activity expressed as nmol/min/mg protein).

2.8. Assay validation

The intra-assay (within-run) precision was assessed by analyzing five replicates of three concentrations of PBM cytosol (100, 150 and 250 μg) on the same day. Inter-assay (between-day) precision was determined by measuring the same controls used for the intra-assay precision study (which was run in duplicate) over five days. The precision is expressed as the coefficient of variation [12] where:

$$\%\text{C.V.} = \frac{(\text{standard deviation})}{\text{mean}} \times 100$$

2.9. Recovery

The mean efficiency of extraction was determined for both pre-filtered and posted filtered samples using

known amounts of [^{14}C]FUra. Recovery of [^{14}C]FUra and [^{14}C]dihydrofluorouracil were evaluated as follows: 20 replicate assays were incubated for 15 min at 37°C under the standard conditions described above [200 μ*M* NADPH, 8.23 μ*M* [6-^{14}C]FUra (56 mCi/mmol), buffer A and 200 μg cytosolic protein (from a healthy donor) in a final volume of 1 ml]. All samples were terminated by adding an equal volume (1 ml) of ice cold 100% ethanol. Five samples were immediately mixed with 6 ml scintillation cocktail and quantitated in a Beckman (Fullerton, CA, USA) LS 6000 liquid scintillation counter. The remaining 15 samples were vortexed and placed in a −70°C freezer for 10 min and then centrifuged for 10 minutes at 14 000 *g*. The supernatant from five samples was mixed with 6 ml scintillation cocktail and the total radioactivity in each sample quantitated as described above. The supernatant from the remaining 10 samples was filtered through a 0.2 μm Acrodisc filter. Five of these samples were then mixed with scintillation cocktail and quantitated. The remaining five samples were injected on the HPLC and quantitated by collecting 1 min fractions and counting the samples in the same liquid scintillation counter described above. Total radioactivity eluted from the columns was determined by measuring the sum of the two peaks.

2.10. Stability of the reaction products

The stability of [^{14}C]FUra and [^{14}C]dihydrofluorouracil in ten terminated reaction mixtures (equal volumes of ethanol and reaction mix) were evaluated over a three month period with samples being stored at −20°C. In addition the stability of ten terminated reaction mixtures was evaluated during storage at room temperature over 48 h.

2.11. Kinetic studies

Kinetic studies were performed on PBM cell cytosol obtained from previously characterized control individuals with normal DPD activity (0.18–0.67 nmol/min/mg). Initial reaction rates were determined for various concentrations of FUra (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.2 μ*M*) in the presence of 200 μ*M* NADPH. Reactions were performed in buffer A (pH 7.4) at 37°C. The incubation time and protein concentration were adjusted so that no more than 20% of the limiting substrate was consumed. Estimation of apparent K_m values was performed by fitting these data to the Michaelis–Menton equation by non-linear regression analysis [13].

3. Results

3.1. Recovery of fura and dihydrofluorouracil from HPLC

This HPLC chromatographic technique unambiguously resolves dihydrofluorouracil from FUra with retention times of 7(±1) and 19(±1) min, respectively, as shown in Fig. 1. In preliminary experiments, multiple injections of known amounts of [6-^{14}C]FUra demonstrated that the total radioactivity applied to the columns was recovered in 25 min (97.5±2.0%) with a coefficient of variation of only 5.5% using the auto-sampler ($n=33$). To determine the amount of sample lost during the ethanol extraction and filtration step of the assay, the recovery of [^{14}C]FUra and [^{14}C]dihydrofluorouracil were evaluated for pre-filtered and post filtered assays as described in Section 2 above (results summarized in Table 1). Briefly, these data demonstrate that less than 5% of the sample is lost during the extraction process.

3.2. Assay validation

Intra-assay precision (%C.V.) for each PBM cytosol protein concentration (100, 150 and 250 μg) tested ($n=5$ for each concentration) did not exceed 6.5%. The inter-assay precision was evaluated over five days using the same protein concentrations described above (100, 150 and 250 μg). Inter-assay variation did not exceed 8%.

3.3. Stability of the reaction products

The mean (±S.D.) ratio of calculated [^{14}C]FUra and [^{14}C]dihydrofluorouracil concentrations (new/old) suggest that the enzyme reaction products are stable in a 50% ethanol mixture for at least three

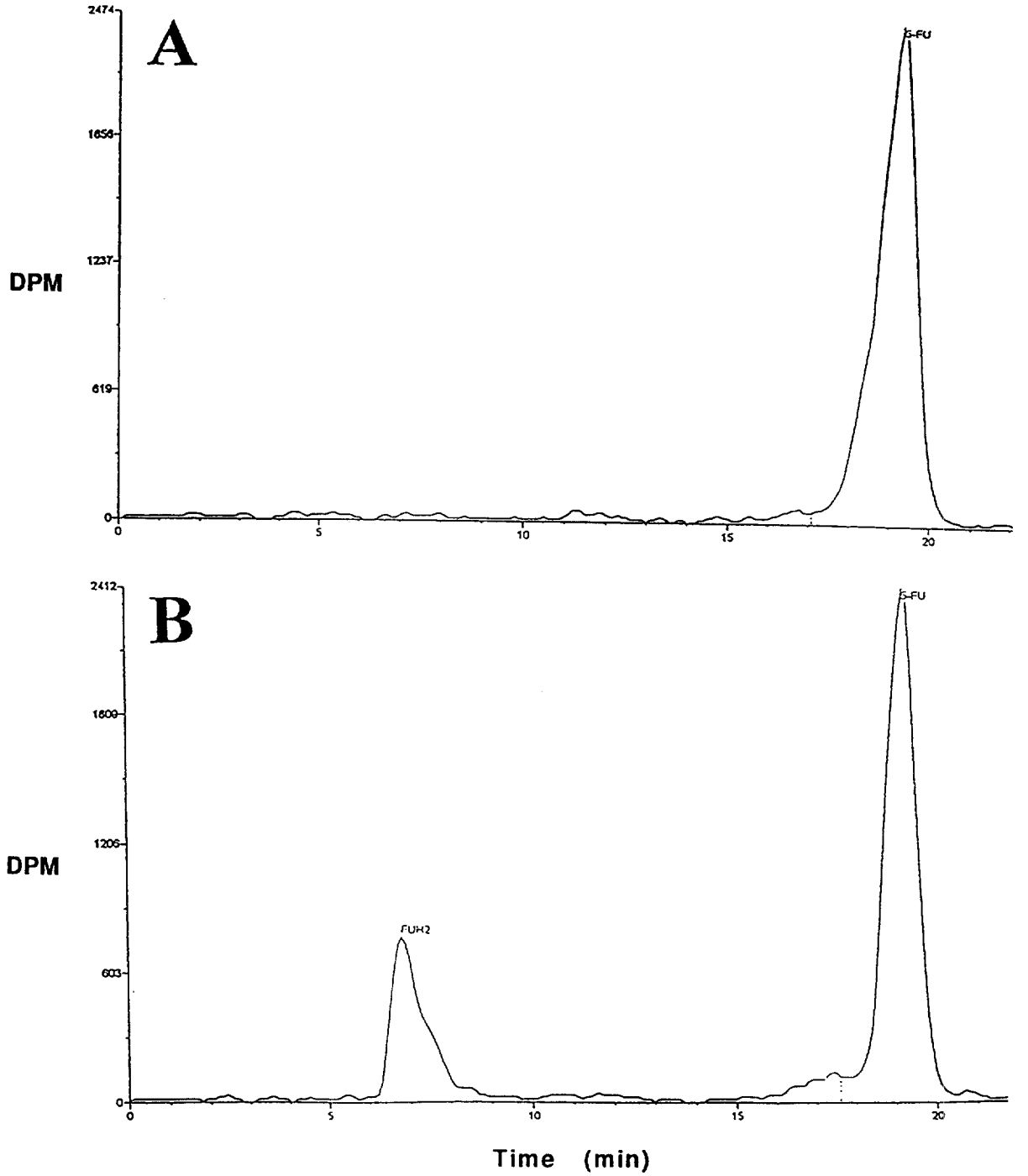

Fig. 1. HPLC elution pattern showing the radioactive profile of (A) [6-^{14}C]FUra and (B) [6-^{14}C]FUra incubated with 250 μg human lymphocyte cytosol for 30 min at 37°C in the presence of 200 μ*M* NADPH in buffer A. Reactions were terminated and injected on HPLC as described in Section 2.

Table 1
Recovery of [^{14}C]FUra and [^{14}C]dihydrofluorouracil for pre-filtered and post filtered assays

Sample ($n=5$)	Mean radioactivity (DPM)	Extraction recovery (%)	Coefficient of variation (C.V.)
Crude assay mixture	1 023 252±2753	100%	0.27%
Ethanol precipitated crude mixture	1 020 114±2567	99.7%	0.25%
Ethanol precipitated filtered	1 004 833±31 150	98.2%	3.1%
Total radioactivity eluted from HPLC (sum of two peaks)	979 252±46 024	95.7%	4.7%

Replicate assays ($n=20$) were incubated for 15 min at 37°C in the presence of 200 μ*M* NADPH, 8.23 μ*M* [6-^{14}C]FUra (56 mCi/mmol), buffer A and 200 μg cytosolic protein (from a healthy donor) in a final volume of 1 ml. Samples were subsequently extracted as described in the Section 2. Total mean radioactivity was determined in the crude assay mixture ($n=5$) and assigned a value of 100%. Recovery from each step in the extraction process is reported as a percent compared to the the total radioactivity determined in the crude assay mixture.

months at −20°C: 1.13 (±0.21) for [^{14}C]FUra ($n=10$) and 0.92 (±0.43) for [^{14}C]dihydrofluorouracil ($n=10$). These data were not affected by at least three freeze/thaw cycles. Evaluation of the 50% ethanol extracts at room temperature demonstrated that both [^{14}C]FUra and [^{14}C]dihydrofluorouracil were stable (no detectable change) over 48 h.

3.4. Optimal conditions and detection limits of the PBM–DPD assay

Previous studies by our laboratory optimized the assay conditions for quantitation of PBM–DPD activity in both fresh and frozen PBM samples [14]. Briefly, these conditions require that the enzyme reaction be maintained at pH 7.4 and 37°C with an NADPH concentration of 200 μ*M*. By controlling the reaction time and the amount of PBM cytosol added to each assay, formation of product was maintained below 20%. Both substrate and product inhibition have been observed with PBM–DPD activity. The minimal detection limit for this assay system was determined to be 0.005 nmol of dihydrofluorouracil.

3.5. Linearity of PBM–DPD assay with time and protein

The rate of dihydrofluorouracil formation was initially examined with respect to incubation time. Human PBM cell cytosol (200 μg) was incubated at 0, 5, 10, 15, 20, 25 and 30 min in the presence of 8.23 nmol [6-^{14}C]FUra in a total volume of 1.0 ml. A linear rate of dihydrofluorouracil formation was observed during the first 30 min of incubation at 37°C (Fig. 2).

To establish whether there was a direct relationship between the amount of protein added and the observed DPD activity, various amounts of human PBM cell cytosol (25–250 μg) were incubated for 15 min. Under these conditions, a linear increase in the rate of dihydrofluorouracil formation was observed (Fig. 3).

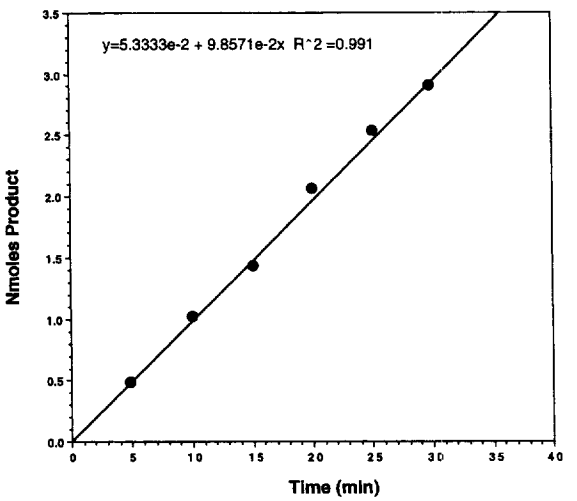

Fig. 2. Production of dihydrofluorouracil was evaluated over a 30 min incubation. All samples contained 200 μg human lymphocyte cytosol, 8.23 nmol [6-^{14}C]FUra and 200 μ*M* NADPH in a total volume of 1.0 ml. Reactions were incubated at 37°C, terminated and injected on HPLC as noted in Section 2.

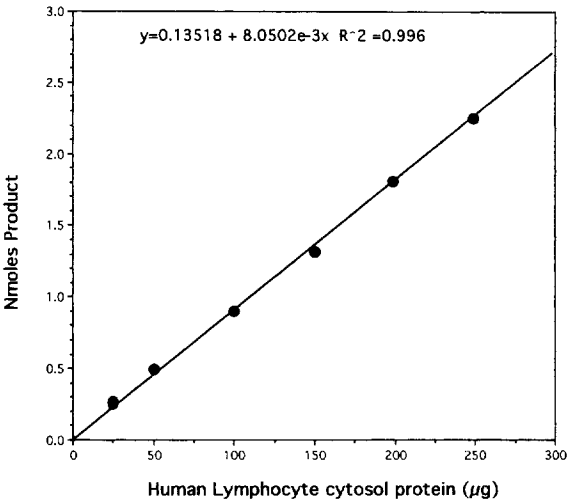

Fig. 3. Production of dihydrofluorouracil in a 15 min incubation with varying amounts of human lymphocytic cytosolic protein (25–250 μg).

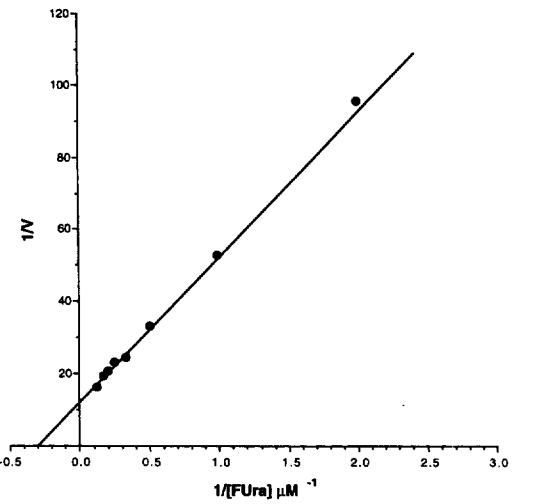

Fig. 4. Double-reciprocal plot of the rate of reaction as a function of the concentration of [6-^{14}C]FUra. The rate of dihydrofluorouracil formation was determined in the presence of 200 μ*M* NADPH using the following concentrations of [6-^{14}C]FUra (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.2 μ*M*).

3.6. Kinetic properties

Previous studies using [^{3}H]pyrimidines showed that uracil, thymine, and FUra were substrates for purified DPD with K_m values of 4.9, 4.8 and 3.3 μ*M*, respectively [15]. Kinetic studies performed using this semi-automated assay system demonstrated that DPD activity varied with increasing pyrimidine concentrations conforming with linear Michaelis–Menten kinetics. These studies produced a K_m value of 3.0 μ*M* for FUra (Fig. 4).

3.7. Population distribution of PBM–DPD activity

Samples from one hundred healthy volunteers (age range 19–58 years) samples were collected from faculty, staff and students from this University who had never been treated with FUra. These data agree with previous studies [10,14] which report that the range of PBM–DPD activity has a normal Gaussian distribution with no differences observed with increasing age. The mean DPD activity in this population was calculated to be 0.431 nmol/min/mg (compared to 0.425 in a previous study [14] by our laboratory of 124 healthy volunteers). The S.D. was calculated to be 0.153 with a S.E. of 0.091.

3.8. New DPD deficient patients

Collaboration with medical oncologists within our university allowed us to use this new assay system to screen cancer patients for DPD activity prior to treatment with FUra. Samples from eighty recently diagnosed cancer patients were obtained during an initial office visit and assayed for DPD activity using the method described above. These data identified two profoundly DPD deficient patients with less than 10% of the mean DPD activity in the general population and below the lower limit of 99% distribution range [14]. A complete characterization of these patients will be presented elsewhere.

4. Discussion

Deficiency of DPD has been shown to be responsible for a pharmacogenetic syndrome in which administration of standard doses of FUra is associated with severe and potentially life-threatening toxicity, including mucositis, granulocytopenia, neuropathy and even death. Since FUra remains one of the most commonly used antineoplastic agents,

determining the level of DPD activity of cancer patients prior to receiving FUra could preclude a DPD deficient patient from potentially fatal toxicity. However, determination of DPD activity of cancer patients prior to beginning chemotherapy has been difficult due to the time consuming, labor intensive methods currently in use [5,7,14].

In the present study, we describe a rapid, sensitive, semi-automated radioassay for measuring DPD activity in human peripheral lymphocytes. This assay was developed to screen for DPD deficiency in high-risk patients, such as those with family members previously demonstrating toxicity to FUra. The principle features of this system are: a HPLC system equipped with an automatic injector allowing multiple sample loading, an in-line flow analyzer for automatic peak detection and a computer software package that automatically converts peak area to nmol of product formed. This system was evaluated for linearity with time and protein concentration and further verified by determining the K_m value for FUra which agreed with previously published results [15,16].

Previously described spectroscopic DPD assay methods measuring NADPH oxidation [16,17] lack specificity; this suggests the possibility, particularly in crude preparations, that the activity of NADPH oxidizing enzymes rather than DPD activity would be measured. Other researchers [18,19] have described specific assays which utilize radiolabelled FUra. These assays separate the reduced product of the reaction (dihydrofluorouracil) by either TLC or HPLC. Samples are then quantitated by autoradiography (for TLC) or by collecting fractions and counting them in a scintillation counter (for HPLC). Unfortunately, these methods are not easily automated, time consuming, expensive and labor intensive.

The clinical diagnosis of DPD deficiency is difficult since the appearance of life threatening toxicity secondary to exposure to FUra is typically the first symptom of this pharmacogenetic syndrome. The aim of this study was to develop a simple, sensitive and accurate method for determining DPD enzyme activity in cancer patients prior to and/or during FUra treatment. When one considers (a) the frequent use of FUra in cancer patients, (b) the severe toxicity to this drug in DPD deficient patients and (c) the potential value of using an individual's DPD activity to attenuate FUra chemotherapy, the benefits of determining a cancer patients DPD activity becomes apparent. Unfortunately, previously described assays for DPD activity are too expensive and labor intensive to be offered in most cancer treatment centers.

Prior to any large scale screening of DPD activity in patients, clear criteria for the identification of deficient patients must be determined.

The precise level of DPD deficiency (or lack of enzyme activity) required to observe toxicity secondary to treatment with FUra remains to be determined. Earlier population studies of PBM–DPD activity in 124 healthy volunteers [14] and 185 cancer patients [10] demonstrated that DPD activity followed a unimodal Gaussian distribution. These studies suggested that toxicity following treatment with FUra occurs when DPD activity levels are below 0.182 and 0.1 nmol/min/mg, respectively. While both these studies agree that complete DPD deficiency is an extremely rare event, neither study attempts to define the limits of DPD deficiency at which toxicity to FUra occurs. One of the clear applications of this new assay method will be to screen enough patients to correlate DPD enzyme activity levels with levels of FUra toxicity and FUra antitumor effectiveness. The DPD assay method described above is a significant improvement on existing methods and is suitable for screening cancer patients for DPD deficiency prior to administration of FUra.

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