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High-performance liquid chromatographic assay with ultraviolet detection for quantification of dihydrofluorouracil in human lymphocytes: application to measurement of dihydropyrimidine dehydrogenase activity

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Abstract

The anticancer drug 5-fluorouracil (5FU) undergoes extensive biotransformation to 5-dihydrofluorouracil (5FUH2) by the enzyme dihydropyrimidine deshydrogenase (DPD). A new HPLC method with direct UV detection for the determination of 5FUH2 in peripheral lymphocytes has been developed to detect DPD deficiency in patients treated with 5FU-based therapy. The method has been shown to be valid over the 5FUH2 concentration range of 1.14–37.88 nmol/ml. Optimal enzymatic conditions for DPD activity measurement were studied: incubation time, protein and 5FU concentrations. The assay was successfully cross-validated with the existing method using HPLC with radiochemical detection.

Keywords: Dihydrofluorouracil; Dihydropyrimidine dehydrogenase; Enzymes

1. Introduction

Dihydropyrimidine dehydrogenase (DPD) deficiency with a defect of the pyrimidine catabolic pathway has recently become the focus of considerable attention, due to severe 5-fluorouracil (5-FU) toxicities occurring in DPD deficiency patients. 5-FU is one of the most widely used anticancer agents in the treatment of breast, head and neck and colorectal cancers [1]. More than 80% of an administered 5FU dose is degraded by DPD, making it an important regulator for this commonly used anticancer drug. Significant inter-individual variations in 5-FU clearance, tumor response and host toxicity have been reported after 5-FU therapy [1–3]. These variations may be due to genetic difference in the activity of DPD enzyme [4]. Results from family studies suggest an autosomal recessive pattern of inheritance of DPD deficiency [5,6]. Based on catalytic activity in population studies, frequency of heterozygotes may be as high as 3% [7] and that of homozygotes could

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be approximately 1/1000 [8]. Patients with low DPD activity have been identified as being at risk for severe toxic reactions to standard doses of 5-FU [7,9]. The level of DPD deficiency related to toxicity is difficult to define precisely. In the different prospective studies published in literature, no case of complete deficiency has been reported [7,10]. However, DPD activity values between 0.100 and 0.150 nmol/min per mg protein can be indicative of an increased risk to more or less severe 5FU related toxicity [11].

Various methods for the detection of DPD deficiency have been developed. The indirect methods are based on the determination of pyrimidine levels in urine [12]. The direct methods are based on the determination of the precise DPD enzymatic activity. To date, the direct measurement of DPD enzyme activity in peripheral lymphocytes by semi-automated radioassay has been the only reliable method to be used in the routine screening of DPD deficient cancer patients [13]. This method requires peripheral blood mononuclear cells (PBMC) isolation and high-performance liquid chromatography (HPLC) analysis of dihydrofluorouracil (5FUH2) formed from [6-^{14}C]5FU. The need for radiolabelled samples, and for a one-line radioisotope flow detector explained why this technique is not applicable for general screening. The development of new, less labour-intensive assays are necessary. In the present study, we reported a new validated HPLC method for measuring DPD enzyme activity in PBMC, using UV detection for quantification of 5FUH2. Its characteristics and its cross-validation with the reference method [13] are described.

2. Experimental

2.1. Chemicals and reagents

5-Fluorouracil (5FU) and dihydrofluorouracil (5FUH2) were obtained from Sigma (St. Louis, MO, USA) and Hoffman La Roche (Basel, Switzerland), respectively. Purified water was purchased from the Milli-Q system (Waters, France). PBMC were separated from whole blood with a lymphocyte separating tube (Leucosep, Dominique Dutscher, Brumath, France). Separating lymphocytes medium (MSL) and phosphate-buffered saline (PBS) were purchased from Eurobio (Les Ulis, France). Sodium dihydrogen phosphate, sodium chloride and glycerol were purchased from Merck (Darmstadt, Germany), sodium hydroxide, sodium sulphate and sulphuric acid from Prolabo (Gradignan, France), sodium phosphate, magnesium chloride and NADPH from Sigma. All reagents were of HPLC grade or equivalent purity. Due to light sensitivity and instability, NADPH was newly prepared before each dosage. The protein assay kit for protein quantification was obtained from Biorad (Ivry sur Seine, France).

2.2. Instrumentation and chromatographic conditions

The assay was developed using a high-performance liquid chromatograph (Waters, France) equipped with a 600 HPLC pump, an automatic injector (Model 717) and a photodiode array detector (Model 996). Data acquisition and processing were accomplished using the Millenium software (Version 3.2, Waters). UV detection was monitored at 205 nm. Chromatographic separation was achieved at 10°C using a Symetryshield RP18 column (5 μm, 4.6 mm×250 mm, Waters, France). Elution was carried out isocratically at a flow-rate of 0.6 ml/mm with a mobile phase consisting of distilled water. One hundred μl of the sample were injected onto the chromatograph system.

2.3. Preparation of calibration standards and quality control samples for 5FUH2 quantification

A stock solution (C=100 μg/ml) for generating the 5FUH2 calibration curve and quality controls was prepared by dissolving an accurately weighed amount in sodium phosphate buffer (35 m*M*, pH 8.0). The solution was then stored in 1-ml aliquots at −20°C. The calibration curve was prepared in the range 1.14–37.88 nmol/ml to encompass the expected concentration of 5FUH2 and DPD enzyme activity in samples. For validation of the assay, calibration standards of six levels (including the blank) and sets of QC samples (four levels) were prepared by adding 5FUH2 stock solution to DPD assay mixture (see Section 2.7) containing no cytosol.

2.4. Assay validation of 5FUH2 determination

A full validation procedure was performed consisting of the following experiments: specificity, linearity, lower limit of quantification (LLQ), limit of detection (LOD), within-day and between-day precisions and accuracy, stability after sample preparation.

The linearity of calibration curves was tested using a weighted linear regression (weighing factor 1/concentration). The peak areas of 5FUH2 were plotted versus the nominal concentrations of the calibration standards. The LLQ was defined as that concentration which can still be determined with acceptable precision (relative standard deviation $<$20%, RSD) and accuracy (percentage of deviation $<$20%, RE). Accuracy and precision of the assay was assessed from the results of six quadruplicate analysis of spiked quality control (QC) prepared at four concentrations (near the LLQ, low, middle range and high of the calibration range). The 5FUH2 concentrations were determined using the standard calibration curve previously validated, and the results were plotted as measured concentrations versus nominal values. The accuracy was defined as the percentage of the ratio of the observed concentration and the nominal concentration. Within-day and between-day precisions were calculated using a one-way analysis of variance (ANOVA) with the analytical run as the group variable. From the ANOVA analysis, the day mean square (DayMS), error mean square (ErrMS) and grand mean (GM) were obtained. Within-day and between-day precisions are calculated by the following formulas:

$$\text{Within-day precision (\%)} = 100 \times \sqrt{\text{ErrMS}}/\text{GM}$$

$$\text{Between-day precision (\%)} = 100 \times \sqrt{\frac{(\text{DayMS}-\text{ErrMS})/N}{\text{GM}}}$$

N is the number of replicates

2.5. Collection of PBMC

Approximately 20 ml of blood were collected into heparinized tubes, transferred in a 50-ml Leucosep tube containing 15 ml of MSL. After centrifugation (10 min, 1100 *g*), the PBMC were removed and washed twice with PBS. Contaminating red blood cells were hypotonically lysed. PBMC were suspended in 35 m*M* sodium phosphate buffer containing 10% glycerol (25×106 cells/ml). The cells suspension was then centrifuged (5 min, 250 *g*) and stored at -80°C until DPD enzyme activity determination.

2.6. Preparation of cytosol

Cells suspension were freeze-thawed, placed in an ice bath and sonicated (three time for 10 s with a 30-s interval between sonication) to lyse the cellular membrane. Following centrifugation at 35 000 rpm for 30 min at $+4$°C, the supernatant was removed and kept on ice until assayed for DPD activity. Cytosolic protein concentrations were determined by the Bradford dye-binding method using γ-globulin as standard.

2.7. DPD assay

The assay consisted of the incubation of 50 μl of cytosol with 5FU (20 nmol/ml final), NADPH (250 μ*M* final) and magnesium chloride (2.5 m*M* final). Total volume was 125 μl (in 35 m*M* sodium phosphate buffer pH 8.0). The duration of incubation was 40 min at 37°C. The reaction was stopped by addition of 125 μl of sulphate sodium saturated solution and acidified by 10 μl of sulphuric acid 2*M* solution. The samples were centrifuged (10 min, 10 000 rpm) to remove proteins and 100 μl of the supernatant was injected in the HPLC system for quantification of 5FUH2 concentrations.

2.8. Calculation of DPD activity

The peak area of 5FUH2 was used to construct the standard curves. The curve was generated by using a $1/x$ weighted least-squares curve fit to the obtained data points. To determine the specific DPD activity of samples, nmol/ml of 5FUH2 formed were expressed as a function of time (min) and divided by

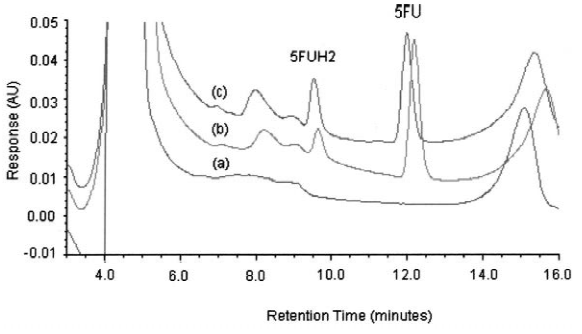

Fig. 1. Chromatograms showing a blank lymphocytes cytosol (a), and 5FUH2 peak in lymphocyte cytosol incubated 15 min (b) and 30 min (c) with 5FU (20 nmol/ml) at 37°C in the presence of NADPH (250 μM).

the amount of protein (mg/ml) added, to obtain the final results.

2.9. Cross-validation with the reference method

The assay was cross-validated by the analysis of PBMC DPD activity obtained from 30 subjects (one healthy and 29 cancer patients), using both HPLC analysis of 5FUH2 formed from [6-^{14}C]5FU and no radiolabelled 5FU. Statistical comparisons of data resulting from cross-validation experiments were performed using the paired *t*-test and regression analysis. Fixed and proportional errors were determined from the 95% confidence limits around the slope and intercept functions of the linear regression. Random error was assessed from the RSD of the individual test data compared to reference data [14].

3. Results

3.1. Assay validation of 5FUH2 determination

3.1.1. Identity

The identity of the peak corresponding in retention time to that of 5FUH2 reference standard was established by two means. (1) The UV spectrum of the putative 5FUH2 peak was matched to that of the reference standard, and (2) the appearance of the 5FUH2 peak occurring during DPD enzymatic reaction incubation (Fig. 1).

3.1.2. Linearity

The slopes and intercepts of the regression curves are listed in Table 1. The linear regression model passed the lack-of-fit test at the level of significance $\alpha = 0.01$. For evaluation of the quality of fit (precision and accuracy), the relative standard deviation (RSD) of the assayed concentrations and the percentage deviation of the assayed concentrations from the nominal concentration (RE) were calculated using data obtained on six consecutive runs. The results (Table 1) indicate excellent linearity in the concentration range of 1.14–37.88 nmol/ml. The criterion for accepting any curve was that all data points should have a RE and a RSD of less than 15%. These criteria were met by the results of the analysis of the lowest calibration standard (1.14

Table 1
Assayed concentrations of calibration standards of 5FUH2

Nominal conc. (nmol/ml)	Assayed concentration (nmol/ml)						Mean	RSD (%)	RE (%)
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6			
1.14	1.15	1.12	1.22	1.27	1.20	1.08	1.17	8.7	0.8
3.79	3.48	3.54	3.82	3.47	3.59	3.82	3.62	4.9	0.1
7.58	7.45	6.86	8.42	8.12	7.27	7.59	7.62	7.9	−0.5
18.94	18.96	17.68	19.94	20.49	17.19	20.25	19.08	7.4	0.3
37.88	38.00	35.90	37.29	39.75	35.90	40.10	37.82	4.9	0.04
Slope	12 109	11 177	12 223	12 799	11 271	12 863			
Intercept	−5438.4	−4277.4	−3056.4	−5151.4	−3216.3	−6678.9			
r	0.9998	0.9994	0.9989	0.9992	0.9997	0.9998			

nmol/ml) where 0.83 and 8.7% were achieved for RE and RSD, respectively.

3.1.3. Sensitivity

The criteria for precision and accuracy at the LLQ were met by the results of the analysis of the calibration standard 1.14 nmol/ml, where 0.83 and 8.7% were achieved for RE and RSD, respectively. The LOD was estimated using a signal-to-noise ratio of 3. The LOD was determined to be 0.60 nmol/ml which corresponds to a DPD enzyme activity comprised between 0.012 and 0.026 nmol/min per mg protein over the protein range (1–2 mg/ml). Thus, the assay could be used for the quantification of 5FUH2 in the reference range [10] of PBMC-DPD activity in cancer patients (0.065–0.559 nmol/min per mg protein).

3.1.4. Intra- and inter-run accuracy and precision

The results are shown in Table 2. Accuracy was less than 8.1% at all levels. Within-day and between-day precisions at each quality control level were always below 10%.

3.1.5. Stability of 5FUH2 after sample preparation

Three QCs were prepared and processed in duplicate at time T0 h with the calibration row. The stability of 5FUH2 in the autosampler at 10°C was investigated by re-analyzing samples at 12 and 24 h with a new calibration row. The results showed no apparent loss of the contained 5FUH2 (data not shown).

3.2. Characteristics of the PBMC-DPD assay

3.2.1. Linearity of PBMC-DPD assay with incubation time

The rate of 5FUH2 formation was studied as a function of incubation time. Fifty μl of lymphocytes cytosol (1.9 mg/ml) were incubated at 0, 5,10,15, 20, 30 and 40 min with 20 nmol/ml of 5FU in a total volume of 125 μl. A linear rate of 5FUH2 formation was observed during the 40-min period studied ($r = 0.995$).

3.2.2. Linearity of PBMC-DPD assay with amount of protein

The rate of 5FUH2 formation was studied as a function of the amount of lymphocyte cytosol protein. Fifty μl of various amounts of lymphocytes cytosol (0.4, 0.9, 1.3, 1.7 and 2.3 mg/ml) were incubated for 35 min with 20 nmol/ml of 5FU. A linear rate of 5FUH2 formation was observed over the range of protein amounts studied ($r = 0.996$).

3.2.3. Kinetic validation

The kinetic study was performed by measuring the rate of 5FUH2 formation as a function of 5FU concentration. Fifty μl of lymphocytes cytosol (1.7 mg/ml) were incubated during 35 min with various concentration of 5FU (0–100 nmol/ml). The amount of 5FUH2 formed and DPD activity varied with increasing 5FU concentrations (Fig. 2) according to linear Michaelis–Menten kinetics ($K_m = 4.0$ nmol/ml and $V_{max} = 17.6$ nmol/ml).

3.3. Cross-validation with the reference method

Thirty samples were collected from one healthy subject and 29 cancer patients and assayed for DPD enzyme activity using both analyses. In the comparison, the method using HPLC analysis of 5FUH2 formed from [6-^{14}C]5FU [13] was designated the *reference* procedure and the method described in the present paper was the *test* method. The results of statistical analysis are shown in Table 3. The mean ratio (test method/reference method) was 1.01 with a RSD of 13.8% and the total assay bias was −3.8%. The paired *t*-test showed no significant difference between the analytical methods. Regression analysis showed confidence intervals around slopes and intercepts consistent with the absence of fixed or proportional biases. The regression curve is shown in Fig. 3.

4. Discussion

The analytical method described here relies on UV-HPLC quantification of 5FUH2. Our assay is

Table 2
Within-day and between-day precisions and accuracy of the quantification of 5FUH2

Nominal QC conc. (nmol/ml)	Within-day precision (%)	Between-day precision (%)	Accuracy (%)	95% C.I. of accuracy[a] (%)
1.14	4.9	8.7	−3.9	−16.6 to 8.8
1.89	9.5	3.6	−7.6	−20.0 to 4.7
11.36	3.2	2.9	−8.1	−13.4 to −2.8
22.73	4.1	2.5	−5.8	−11.7 to 0.16

[a] 95% CI, 95% confidence interval.

similar to that reported by Johnson et al. [13], although we detected 5FUH2 formed from 5FU with direct UV detection at 205 nm. Johnson et al. [13] used a radio-HPLC assay for quantification of 5FUH2 formed from [6-^{14}C] 5FU. However, this latter technique is limited in our opinion by the inconvenience of carrying out the radioactive step in the context of a large laboratory development.

Good separation of 5FUH2 from 5FU was observed using the current chromatographic conditions. Linearity of 5FUH2 calibration curves was excellent over the anticipated concentration range with correlation coefficient of >0.9989. The standard curves obtained during the six validation runs demonstrated linearity over the range of concentrations studied. Precision and accuracy of the low concentration range were improved by using the weighted $(1/x)$ linear regression method. Within-day and between-day precisions and accuracy did not exceed 10% variation at low and high concentrations. These values were comparable than those of the method described by Johnson et al. [13], who reported intra- and inter-assay precision of 6.5 and 8.0%, respectively. The limit of detection and quantification were 0.60 and 1.14 nmol/ml, respectively. The sensitivity of the assay is lower than the reference study but is sufficiently sensitive for the determination of patient PBMC-DPD activity ranging from 0.065 to 0.559 nmol/min per mg protein (mean activity±SD found in 185 cancer patients: 0.222±0.084 nmol/min per mg protein) [10].

Application of our assay was demonstrated by the cross-validation analysis of samples from 30 sub-

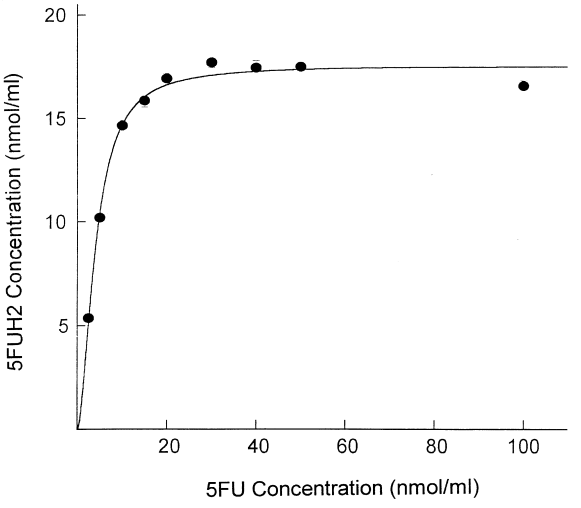

Fig. 2. Formation of 5FUH2 as a function of 5FU concentrations. The reaction was determined in the presence of 250 μ*M* NADPH in a total volume of 125 μl, using the following concentrations of 5FU (2.5, 5,10, 15, 20, 30, 40, 50, 100 nmol/ml).

Table 3
Summary of statistics observed in the cross-validation of radioisotope and UV detection of 5FUH2 for the determination of DPD activity in human lymphocytes cytosol

Test	Statistic
Ratio analysis	
Mean ratio (test method/ reference method	1.01
RSD	13.8%
Paired t-test	
Observed *t*	0.66
t-Critical ($P=0.05$)	1.96
Regression analysis	
Intercept	−0.0061
(confidence interval)	−0.038 to 0.026
Slope	1.009
(confidence interval)	0.89 to 1.12
Fixed bias (reference/test)	Not significant
Proportional bias (reference/test)	Not significant

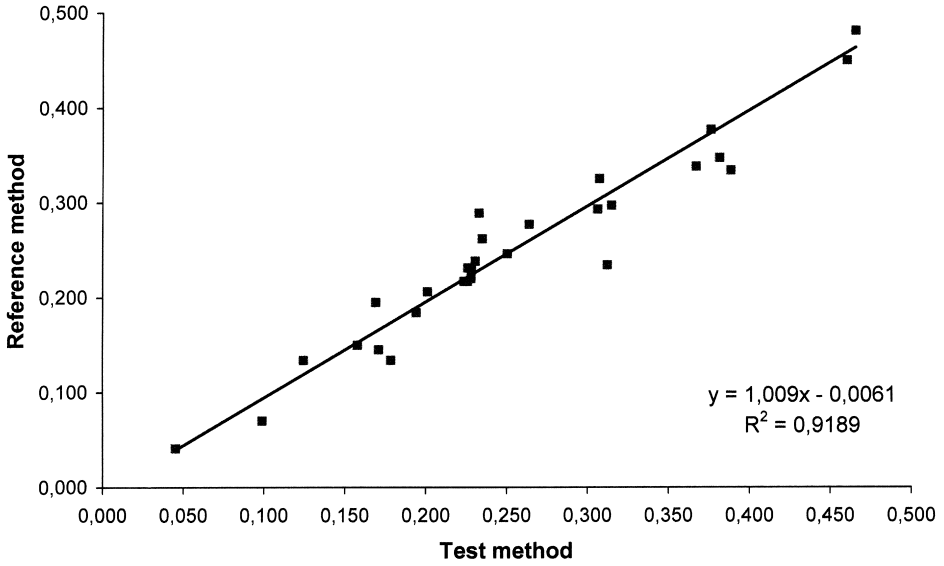

Fig. 3. Cross-validation of assays for the determination of DPD activity (nmol/min per mg protein) in human lymphocytes cytosol using the in-line radioisotope flow detection (*reference* method) and the UV detection (*test* method) of 5FUH2.

jects. The results of the experiments showed no significant difference between the reference and the test methods and no fixed or proportional biases. Based on these results, we concluded that the two methods yield identical results.

In summary, the method met all acceptance criteria in term of linearity, accuracy and precision. The sensitivity is sufficient to monitor DPD activity in patients. Compared to previously employed radio-HPLC assay, this new technique obviates the need for radiolabelled 5FU. This new assay could be ideal for monitoring DPD activity in patients treated with 5FU-based therapy, due to the availability of UV detectors in most hospital laboratory equipped with current HPLC.

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