Focus on fluorouracil
A simple and sensitive fully validated HPLC-UV method for the determination of 5-fluorouracil and its metabolite 5,6-dihydrofluorouracil in plasma

Jan Gerard Maring¹, Leonie Schouten², Ben Greijdanus³, Elisabeth G.E. de Vries⁴, Donald R.A. Uges³

¹Department of Pharmacy, Diaconessen Hospital Meppel and Bethesda Hospital Hoogeveen; ²University Centre for Pharmacy, Groningen; Departments of ³Pharmacy and ⁴Medical Oncology, University Hospital Groningen, The Netherlands

Therapeutic Drug Monitoring, in press
Chapter 3

HPLC-UV analysis of 5-fluorouracil

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Abstract

**Aim** The authors developed a simple and sensitive, fully validated HPLC-UV method for the determination of both 5-FU and its metabolite DHFU in small volume plasma samples.

**Methods** The analytes were separated on a 4.6 X 250 mm I.D. Atlantis dC18 5 µm column, with isocratic elution at room temperature. Chlorouracil was used as internal standard. The analytes were detected with an UV diode array detector. DHFU was detected at 205 nm, 5-FU at 266 nm and chlorouracil at both wavelengths.

**Results** The limits of quantification in plasma were 0.040 µg/mL for 5-FU and 0.075 µg/mL for DHFU. Linearity, accuracy, precision, recovery, dilution, freeze-thaw stability and stability in the sample compartment were evaluated. The method appeared linear over a range from 0.04-15.90 µg/mL for 5-FU, and from 0.075-3.84 µg/mL for DHFU.

**Conclusions** The method appeared very suitable for therapeutic drug monitoring and pharmacokinetic studies of 5-FU, due to the simple extraction and the small sample volume. Problems in earlier published methods with interfering peaks and variable retention times were overcome. The method appeared also suitable for detection of uracil and its metabolite dihydrouracil in plasma.

Introduction

5-Fluorouracil (5-FU) is a fluoropyrimidine anti-cancer agent that was introduced more than 40 years ago [1]. It belongs to the class of antimetabolites and is still widely used in the treatment of gastrointestinal-, breast- and head and neck cancer [2]. Before 5-FU can exert its cytotoxic effects, intracellular metabolic activation is a prerequisite. The principal cytotoxic metabolite of 5-FU is 5-fluoro-2'-deoxyuridine monophosphate (FdUMP), which inhibits thymidylate synthase. This results in inhibition of DNA-synthesis due to depletion of thymidine nucleotides [1,3]. The cytotoxicity, though, is caused by only a small part of the 5-FU dose, since the majority of 5-FU is rapidly metabolized into inactive metabolites. The initial and rate-limiting step in the catabolism of 5-FU is the reduction of 5-FU into 5,6-dihydrofluorouracil (DHFU) by the enzyme dihydropyrimidine dehydrogenase (DPD) [1,3]. As a result, the pharmacokinetic profile of 5-FU displays rapid distribution and rapid elimination [3,4]. Reported half-lifes range from 6 to 22 min [1,3,4-7]. This large variation in 5-FU clearance is thought to be due to interindividual differences in DPD activity. Unfortunately, DPD activity is largely reduced in 1-2% of the Caucasian population due to a mutation in the gene encoding DPD [8-11]. When these people are treated with 5-FU, they risk extreme toxicity due to a largely reduced 5-FU clearance. Since 5-FU based chemotherapy is one of the cornerstones of colorectal cancer treatment, combined with the fact that relatively high 5-FU dosages are applied in this cancer, extreme 5-FU toxicity due to DPD deficiency most frequently is encountered in colorectal cancer patients.
Today, DPD deficiency is recognized as a serious contraindication for 5-FU treatment, and as a result, several methods so far have been evaluated for early detection of DPD deficiency. DPD activity measured in peripheral blood mononuclear cells (PBMC-DPD) has been proposed as screening tool to detect DPD deficiency, but the correlation between PBMC-DPD activity and 5-FU clearance seems to be rather weak [10,12,13]. Alternatively, monitoring of the 5-FU/DHFU ratio in plasma might be a reliable predictor for 5-FU clearance, although this requires the administration of at least one 5-FU dose and the availability of a rapid screening method for 5-FU plasma levels to guarantee reporting on aberrant pharmacokinetics before the second gift is administered [13-16].

Several methods for 5-FU and DHFU detection in plasma have been published [4,7,17-21]. To date various HPLC methods with UV detection have been developed, but most of these methods lack adequate sensitivity for pharmacokinetic studies or therapeutic drug monitoring, since 5-FU plasma levels during 5-FU protracted infusion or after administration of oral 5-FU analogues are generally low [19,21]. Difficulties in the determination of 5-FU and DHFU in plasma with HPLC-UV are mainly due to the hydrophilicity of the analytes, causing variable retention on C18-columns, and due to the limited UV-absorption of DHFU. The HPLC method most suitable for drug level monitoring, published up to now is, the method of Ackland et al., because of its low limits of quantification (LLQ) [17]. It requires, however, a relatively large sample volume and the method has some analytical interference. Therefore we developed an analytical method for the simultaneous determination of 5-FU and DHFU in small volume plasma samples, with sufficiently low limits of quantification for both substances.

Materials and methods

Chemicals
5-Fluoro 5,6-dihydrouracil (95%) was provided by Roche (Basel, Switzerland). 5-Fluorouracil and the internal standard 5-chlorouracil were obtained from Sigma (Zwijndrecht, The Netherlands). Methanol (HPLC grade), ethylacetate and acetonitrile (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ultra pure water was used in all preparations (Milli-Q water purification system, Millipore Benelux; Etten-Leur, The Netherlands). All other chemicals were of analytical grade. Human EDTA-plasma for standard and control samples was provided by the Red Cross Bloodbank (Groningen, The Netherlands).

Chromatographic equipment
Analysis of 5-FU and DHFU was performed on a HPLC-system consisting of a Merck Hitachi L-7110 isocratic pump (Merck, Darmstadt, Germany), a Merck Hitachi L-7200 autosampler and a Merck Hitachi L-7450 UV PDA detector. Integration was performed by Merck Hitachi
Model D7000 HPLC System Manager version 3.1.1 (1994-1999). The system and column operated at room temperature (19-22 °C). The separations were performed on a Atlantis dC18 (4.6 x 250 mm I.D.) 5 µm column (Waters, Etten-Luer, The Netherlands), equipped with a Phenomenex C18 (5 µm, 4.0 x 3.0 mm) SecurityGuard guard column (Bester, Amstelveen, The Netherlands). The mobile phase consisted of 990 mL 1.5 mM phosphate buffer (pH 5.8) mixed with 10 mL methanol. The flow during the analysis was 0.8 mL/min. Spectra were acquired in the 200-300 nm range up to 23 min after injection. The time between the injections was 22 min, to allow the elution of strongly retained endogenous compounds. The injection volume was 50 µL.

**Preparation of the plasma standards and controls**
For each compound, except chlorouracil (internal standard), two independent stock solutions were prepared in water. One was used for the preparation of the calibration samples, the second for the preparation of the validation samples. The 5-FU stock solution contained 100 µg/mL, the DHFU stock solution 25 µg/mL. The concentration of the chlorouracil internal standard stock solution was 200 µg/mL. An internal standard working solution (2 µg/mL) was freshly prepared on the day of analysis by diluting the stock solution 100 times with water. Calibration samples were prepared by adding the required amount of stock solution to human plasma to obtain concentrations of 0.04; 0.08; 0.20; 0.32; 0.40; 0.80; 1.60; 4.80 and 15.90 µg/mL 5-FU, and concentrations of 0.08; 0.10; 0.20; 0.40; 0.80; 1.00; 1.20; 2.00 and 3.84 µg/mL DHFU. Calibration samples of 5-FU and DHFU were combined. Validation samples were prepared at low, medium and high concentration levels and contained at low level 0.092 µg/mL 5-FU plus 0.132 µg/mL DHFU, at medium level 0.80 µg/mL 5-FU plus 0.48 µg/mL DHFU, and at high level 4.00 µg/mL 5-FU plus 0.96 µg/mL DHFU. All standards, controls and stock solutions were stored at -20 °C until use.

**Sample preparation**
An aliquot of 200 µl plasma sample was mixed with 50 µl internal standard working solution in an Eppendorf microfuge tube. Subsequently, 150 µl acetonitril was added, followed by vortex mixing for 10 s. The samples were centrifuged at 11,000 g for 5 min at room temperature. The supernatant was transferred into a glass centrifuge tube and 7 mL of ethylacetate was added. The samples were extracted during 15 min in a rotary mixer. Subsequently, the samples were centrifuged at 3,000 g for 5 min. The organic upper layer was transferred into a conical tube and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The samples were reconstituted in 100 µL mobile phase and transferred into a glass insert for auto-sampler vials. A 50 µL aliquot was injected into the HPLC.
5-FU pharmacokinetics
A plasma sample was obtained from a colorectal cancer patient who was evaluated for DPD deficiency. The patient had experienced more than expected toxicity during the first cycle of a 5-day 5-FU/LV Mayo Clinics schedule. The clearance of 5-FU was evaluated on the first day of the second cycle. A single blood sample was collected at t=60 min after a 5 min intravenous infusion of 380 mg/m$^2$ 5-FU (80% from normal 425 mg/m$^2$ dose). The samples were placed on ice immediately after collection. Analysis of the plasma sample was performed on the same day.

Validation procedure
The method was validated on linearity, accuracy, recovery, freeze-thaw stability, sample compartment stability and sample dilution. On day one, the linearity of the calibration curves and the stability in the sample compartment were determined. On the days two to six, precision and accuracy, recovery, freeze/thaw stability and dilution of samples were tested. During validation, six blank samples obtained from six different human volunteers were tested to demonstrate that there were no interfering components. The results of the tests were evaluated against international used acceptance criteria described by Shah et al [22].

Linearity, Accuracy, and Recovery
To evaluate linearity of the calibration curves three calibration curves were prepared and analyzed. The curves were judged linear if the correlation coefficient $r$ was better than 0.99 as calculated by weighted linear regression. The goodness of fit and the lack of fit were determined by ANOVA calculations.

To assess the accuracy and precision of the method, the samples LLQ, low, medium and high were analyzed three times a day, during five days.

To determine the accuracy, the average bias was calculated. The Relative Standard Deviation (RSD%) was estimated by ANOVA, and used to determine the within-run and between-run precision. Relative standard deviations and biases of less than 15% were accepted, except for the LLQ. For the LLQ relative standard deviations and biases of less than 20% were accepted.

Accuracy was calculated from the mean of the amount observed and the theoretical concentrations at a particular level. The method was considered accurate when the deviation from the theoretical concentration (bias) was less than 20 % at the LLQ level and less than 15 % at the remaining levels [22].

The recoveries were determined by comparing the peak heights of the validation samples low, medium and high with the peak heights of analyzed solutions containing all the compounds of interest at a concentration corresponding with 100 % recovery.
Freezing and Thawing
To assess freezing and thawing stability samples with a low concentration and samples with a high concentration were prepared. The same sample was frozen and thawed each day for five days. After each freeze/thaw cycle the samples were analyzed in triplicate. Bias was calculated in the same way as with precision and accuracy. Samples were considered stable if bias was below 10% after five freeze/thaw cycles.

Dilution
Because some samples of clinical patients can have concentrations above the higher limit of quantification (HLQ), these samples have to be diluted to obtain concentrations within the calibration range. The effect of sample dilution was tested with plasma control samples spiked with 20.00 µg/mL 5-FU plus 4.80 µg/mL DHFU. These samples were diluted 5 times in triplicate on 5 different days. Blank human plasma was used as diluting agent. A bias of less than 10% between the measured undiluted concentration and nominal undiluted concentration was accepted.

Stability during storage in sample compartment
Stability during storage in sample compartment was determined by analyzing samples with low and high concentration over a period of 30 h. The calculated response at t = 30 h was compared to the calculated response at t = 0 h. Samples were judged stable over a period of 30 h if the decrease in response was less than 10%.

Results
Retention times
The retention time of DHFU was approximately 8.2 min. 5-FU and chlorouracil eluted at approximately 10.1 and 19.9 min respectively. Interfering peaks were found in none of six different blank plasma samples, except for a small peak at approximately 19.8 min, close to the peak of the internal standard. Since this small matrix peak turned out to be always at least 30 times smaller than the internal standard peak, it was considered negligible. One other matrix peak could potentially interfere with the DHFU peak (see figure 1 in lower chromatogram at approximately 7.3 min), but this problem was easily overcome as only the retention time of this interfering substance and not that of DHFU appeared dependent on the pH of the mobile phase. The pH can at least vary from 5.7 through 6.3, without affecting the retention times of the peaks of interest. Interference from strongly retained matrix components in subsequent chromatograms was prevented by introduction of a 22 min delay after each injection. We recommend to flush the column with 50% methanol in water for 30 minutes every 3 series of samples, as the column may retain matrix components.
Figure 1 Representative chromatograms of patient plasma sample at \( t=60 \) min after the start of a 5 min infusion of 380 mg/m\(^2\) 5-FU. The upper chromatogram was recorded at 266 nm, the lower at 205 nm. The retention time of DHFU was 8.11 min, the retention time of 5-FU 10.21 min and chlorouracil (internal standard) eluted at 20.17 min.
Linearity, Accuracy and Recovery

The correlation coefficient of the calibration curves was always better than 0.99 for both components. All curves showed no significant lack of fit and for all calibration curves the Goodness of Fit was significant. Table 1 lists the within- and between-run relative standard deviations and the biases of 5-FU and DHFU. All results were within validation limits. The mean recoveries of 5-FU at low, medium and high concentrations were 75.3%, 77.6% and 81.1% respectively (n=15). For DHFU the values were 65.5%, 68.3% and 70.5%. For chlorouracil a recovery of 74.2% was found.

Dilution

Bias was below 10% for both 5-FU and DHFU. Detailed results are listed in table 2.

Table 1  Accuracy and within-run and between-run precision of 5-FU and DHFU in plasma in different concentrations (n=15).

<table>
<thead>
<tr>
<th></th>
<th>Nominal concentration (µg/mL)</th>
<th>Measured concentration (µg/mL)</th>
<th>Mean bias (%)</th>
<th>Within-run RSD (%)</th>
<th>Between-run RSD (%)</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>0.040</td>
<td>0.043</td>
<td>+6.3</td>
<td>3.7</td>
<td>3.5</td>
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<tr>
<td></td>
<td>0.092</td>
<td>0.099</td>
<td>+7.9</td>
<td>2.9</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>0.802</td>
<td>0.831</td>
<td>+3.7</td>
<td>2.7</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>4.090</td>
<td>4.260</td>
<td>+4.2</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>DHFU</td>
<td>0.075</td>
<td>0.087</td>
<td>+15.6</td>
<td>3.2</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>0.132</td>
<td>0.134</td>
<td>+1.5</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>0.480</td>
<td>0.478</td>
<td>–0.3</td>
<td>3.4</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>0.959</td>
<td>0.967</td>
<td>+0.8</td>
<td>1.2</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Table 2  Biases for the dilution test

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Nominal undiluted concentration (µg/mL)</th>
<th>Measured concentration (µg/mL)</th>
<th>Bias (%)</th>
<th>Within-run RSD (%)</th>
<th>Between-run RSD (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FU</td>
<td>20.00</td>
<td>20.80</td>
<td>+4</td>
<td>2.3</td>
<td>4.4</td>
<td>15</td>
</tr>
<tr>
<td>DHFU</td>
<td>4.80</td>
<td>4.63</td>
<td>–4</td>
<td>3.6</td>
<td>4.4</td>
<td>15</td>
</tr>
</tbody>
</table>
Freeze-thaw stability

Figure 2 depicts the results of the freeze-thaw stability test. The biases for 5-FU were less than 10% during all cycles at both concentration levels. For DHFU, at the 0.142 µg/mL concentration level, the bias exceeded 10% after four freeze-thaw cycles. At the 0.921 µg/mL level, bias overstepped 10% after three freeze-thaw cycles. This implicates that DHFU is only stable for two freeze-thaw cycles.

Stability in the sample compartment.

For 5-FU, in low concentration (0.092 µg/mL), the peak height ratio is stable during 20 hours, but at 30 hours this ratio dropped 31.2%. At 8.19 µg/mL 5-FU the peak height ratio increased linearly 2.3% during 30 hours. For DHFU a linear decrease in peak height ratio was observed at both concentration levels. After 30 hours, the decrease was 11.6% at the 0.132 µg/mL level, and 2.7% at 1.92 µg/mL.

Human plasma samples

Figure 1 depicts the chromatograms of a plasma sample at t = 60 min after an intravenous dose of 380 mg/m² 5-FU. The calculated amounts were 0.45 µg/mL for 5-FU and 3.43 µg/mL for DHFU. These values are within the normal range. The patient was not considered DPD deficient.

**Figure 2** Freeze-thaw characteristics of 5-FU and DHFU during 5 cycles. The 5-FU concentrations at the start of the experiment at low (0.097 µg/mL) and high (4.07 µg/mL) concentration levels were not affected after 5 freeze-thaw cycles. However, the DHFU concentrations at both low (0.142 µg/mL) and high (0.921 µg/mL) concentration levels were largely decreased by freeze-thawing.
Discussion

In this paper, we present a fully validated, relatively simple method for sensitive detection of 5-FU and its metabolite DHFU in small volume plasma samples. The assay procedure was based on a method previously described by Ackland et al. for extraction of 5-FU and DHFU from 1 mL plasma samples [17]. We wished to improve this method with regard to extraction procedure and sample size. We discovered that the pH of the aqueous layer can vary freely between 2 and 8 without affecting the extraction efficiency. Therefore pH stabilization with an extraction buffer was considered unnecessary. We managed to simplify the extraction procedure by using acetonitril in stead of trichloroacetic acid 50% for protein denaturation. That disposed of the need for a neutralization step and, as a result, kept the volume of aqueous phase relatively small, around 350 µL. In the Ackland method, the aqueous phase comprises about 2 mL after acid neutralization with sodium acetate. Because of this relatively large volume of aqueous layer, they needed to perform a double extraction procedure with twice 5 ml ethylacetate to obtain recoveries above 70%. The 7 mL ethylacetate used in our procedure is 20-fold in excess of aqueous material and this resulted in recoveries above 75% in a single extraction step. Evidently, the ratio of the volumes of aqueous and ethylacetate layers during extraction appears to be a determinant factor with large impact on the recovery. This is confirmed by data of Loos et.al. who applied an ethylacetate : aqueous layer ratio above 25 in their assay for 5-FU and thus gained recoveries of about 90% [23]. They could, however, omit a deproteinization step, as the dihydrofluorouracil metabolite was not included in their assay. Due to a deproteinization step, our aqueous layer is somewhat larger. We decided not to increase the amount of ethylacetate above 7 mL, in order to maintain the extraction as single-step procedure. Our validation results show that a recovery of about 70% is large enough to obtain accurate and precise results.

5-FU turned out to be stable for at least five freeze-thaw cycles. DHFU however appeared stable for only 2 freeze-thaw cycles. Previous reports confirm the observed instability of DHFU in plasma at room temperature [24]. Therefore, it has been recommended to place blood samples on ice and to centrifuge blood shortly after collection. If reanalysis of a plasma sample is needed, it has been proposed to use only once-thawed samples. The observed freeze-thaw instability has also implications for the conditions during transport: it is important to transport plasma samples on dry ice, to prevent thawing.

The instability of DHFU also has consequences for the storage of samples in the autosampler compartment. At low concentrations 5-FU and DHFU appeared stable for no longer than 20 hours at ambient temperature. This implicates that sample runs may not last longer than 20 hours. Moreover, samples expected to have a low concentration of 5-FU or DHFU should be placed more forwards in the autosampler compartment. Cooling of the autosampler compartment is recommended if available on the equipment. The cause of the non-linear decrease of the 5-FU concentration that we observed during storage in the
autosampler is unknown. It could be the result of auto-inducible deterioration or adsorption to the wall of the vials, but we couldn’t prove these hypotheses.

Our method so far has been applied to clinical research samples and therapeutic drug monitoring of 5-FU in colorectal cancer patients. A very common colorectal cancer treatment implies the administration of a bolus dose of 425 mg/m\(^2\) 5-FU plus 20 mg/m\(^2\) folinic acid on 5 consecutive days (total 5-FU dose 2125 mg/m\(^3\)) in a 28 day cycle (Mayo Clinic schedule). A large proportion of all colorectal cancer patients is treated this way, at least in most Anglo-Saxon countries. From previous work we learned that the 5-FU plasma concentration 5 min after a 5-FU bolus dose of 425 mg/m\(^2\) can range from 30 to 60 µg/mL [25]. With linearity demonstrated up to 15.9 µg/mL and five times dilution possible these concentrations are covered with the current method. The peak plasma concentration of DHFU after the same dose of 5-FU normally ranges from 2-6 µg/mL [25]. This concentration range is also covered by our method with linearity of DHFU demonstrated up to 3.84 µg/mL. The low LLQ values (0.04 µg/mL for 5-FU and 0.075 µg/mL for DHFU) make it even possible to measure plasma levels during 9-10 half-lifes after 5-FU administration. As the plasma half-life of 5-FU is about 10 min, blood levels can be monitored until 90-100 min after administration of a typical 5-FU dose of 425 mg/m\(^2\), which is convenient for most research purposes. Moreover, the current method also appeared very suitable for therapeutic drug monitoring of 5-FU. In an earlier study in 33 clinical patients, we showed that the 5-FU plasma levels at t=1 h after an intravenous bolus dose of 425 mg/m\(^2\) 5-FU ranges from 0.1 – 2.5 µg/mL (mean value 0.6 µg/ml) [25]. The upper level of 2.5 µg /ml corresponds with the mean + 3SD value. Thus, in 99% of all individuals with normal DPD activity, 5-FU levels below this value can be expected. Based on these calculations we consider levels above 2.5 µg/mL at t=1 h after a standard 425 mg/m\(^2\) 5-FU bolus dose as atypical and suspect DPD deficiency in such patients. Previously we have shown that in a patient with heterozygosity for a IVS14+1G→A splice site mutation in the DPD encoding gene, the 5-FU level at t=1 h was 10.0 µg/mL, which is indeed higher than our proposed cut-off level of 2.5 µg/mL [11]. This particular patient suffered from extreme toxicity after a normal 5-FU treatment.

We acknowledge that the major drawback of our approach is the fact that it implies a retrospective evaluation of DPD activity status. Unfortunately, good alternatives are lacking at this moment.

Mattison et al. developed a simple, however expensive, uracil breath test for DPD phenotyping, based on the release of \(^{13}\)CO\(_2\) from 2-\(^{13}\)C uracil in the presence of intact DPD [26]. Expired air was collected 5-90 min after oral ingestion of 6 mg/kg 2-\(^{13}\)C uracil. Partially deficient DPD breath profiles were well differentiated from normal profiles. An oral challenge with uracil, prior to chemotherapy, with subsequent measurement of the uracil clearance in plasma, might be an alternative approach for DPD phenotyping. Therefore, we are currently testing the applicability of our method for uracil detection. More research
is needed to explore, optimize and validate this approach. The current method has further proven its suitability and stability in a phase II pharmacokinetic study of a new investigational cytotoxic agent tested in combination with 5-FU and folinic acid.
References

17. Ackland SP, Garg MB, Dunstan RH. Simultaneous determination of DHFU and 5-fluorouracil in


