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Development of an LC–MS/MS assay for the quantitative determination of the intracellular 5-fluorouracil nucleotides responsible for the anticancer effect of 5-fluorouracil

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ABSTRACT

5-Fluorouracil (5-FU) and its oral prodrug capecitabine are among the most widely used chemotherapeutics. For cytotoxic activity, 5-FU requires cellular uptake and intracellular metabolic activation. Three intracellular formed metabolites are responsible for the antineoplastic effect of 5-FU: 5-fluorouridine 5′-triphosphate (FUTP), 5-fluoro-2′-deoxyuridine 5′-triphosphate (FdUTP) and 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP).

In this paper, we describe the development of an LC–MS/MS assay for quantification of these active 5-FU nucleotides in peripheral blood mononuclear cells (PBMCs). Because the intracellular 5-FU nucleotide concentrations were very low, maximization of the release from the cell matrix and minimization of interference were critical factors. Therefore, a series of experiments was performed to select the best method for cell lysis and nucleotide extraction. Chromatography was optimized to obtain separation from endogenous nucleotides, and the effect of different cell numbers was examined.

The assay was validated for the following concentration ranges in PBMC lysate: 0.488–19.9 nM for FUTP, 1.66–67.7 nM for FdUTP and 0.748–30.7 nM for FdUMP. Accuracies were between −2.2 and 7.0% deviation for all analytes, and the coefficient of variation values were ≤4.9%.

The assay was successfully applied to quantify 5-FU nucleotides in PBMC samples from patients treated with capecitabine and patients receiving 5-FU intravenously. FUTP amounts up to 3054 fmol/10^6 PBMCs and FdUMP levels up to 169 fmol/10^6 PBMCs were measured. The FdUTP concentrations were below the lower limit of quantification. To our knowledge, this is the first time that 5-FU nucleotides were quantified in cells from patients treated with 5-FU or capecitabine without using a radiolabel.

1. Introduction

The anticancer drug 5-fluorouracil (5-FU) has been in clinical use for more than 50 years and is still one of the most widely used chemotherapeutics. Together with its oral prodrug capecitabine, 5-FU has remained the mainstay in the treatment of a range of cancer types, including those originating in the gastrointestinal tract, breast and head and neck region.

5-FU is a uracil analog with a fluorine atom at the C-5 position in place of a hydrogen. For cytotoxic activity, 5-FU requires cellular uptake and metabolic activation by cellular phosphorylases and kinases (Fig. 1). Based on experiments with radiolabeled 5-FU, three of the intracellular formed metabolites (nucleotides) are responsible for the antineoplastic effect of 5-FU [1]. These metabolites are 5-fluorouridine 5′-triphosphate (FUTP), 5-fluoro-2′-*deoxy*uridine 5′-triphosphate (FdUTP) and 5-fluoro-2′-*deoxy*uridine 5′-monophosphate (FdUMP; Fig. 2). In brief, **FUTP** is incorporated into RNA and interferes with normal RNA processing and function. **FdUTP** is incorporated into DNA, leading to pathological DNA structures and ultimately cell death. **FdUMP** inhibits thymidylate synthase, the enzyme that catalyzes the transformation of *deoxy*uridine monophosphate (dUMP) to *deoxy*thymidine monophosphate (dTMP). Inhibition of thymidylate synthase by FdUMP leads to accumulation of dUTP and depletion of dTTP. This

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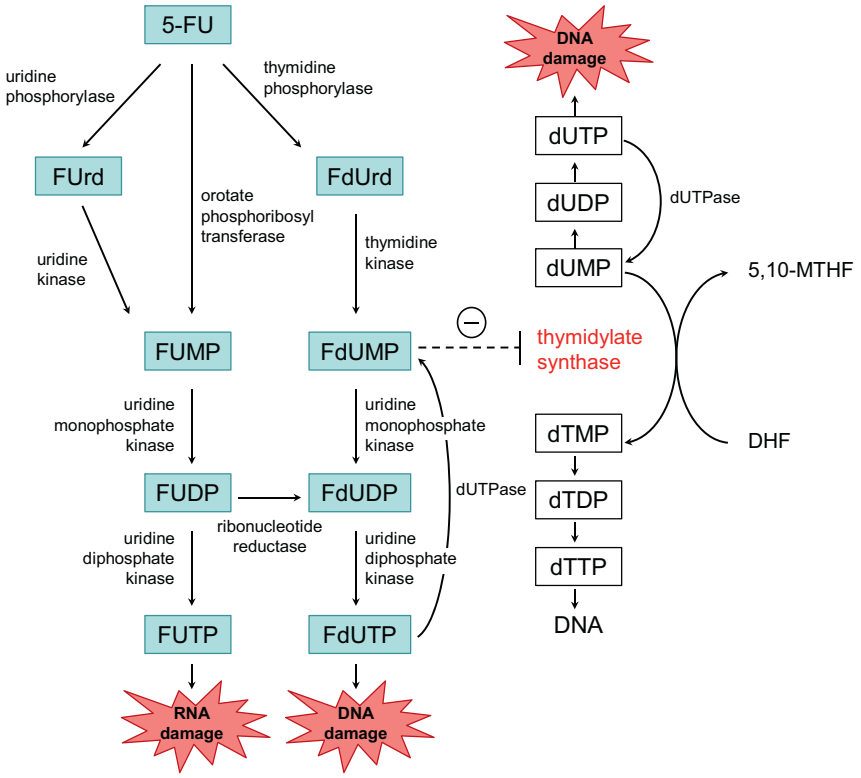

Fig. 1. Intracellular metabolism and mechanisms of action of 5-fluorouracil. This figure shows only the *anabolic* route, which gives rise to the active metabolites. The *catabolic* route, which inactivates 5-FU and leads to elimination of the drug, is omitted.
Abbreviations: 5-FU, 5-fluorouracil; FUrd, 5-fluorouridine; FdUrd, 5-fluoro-2′-*deoxy*uridine; FUMP, 5-fluorouridine 5′-monophosphate; FUDP, 5-fluorouridine 5′-diphosphate; FUTP, 5-fluorouridine 5′-triphosphate; FdUMP, 5-fluoro-2′-*deoxy*uridine 5′-monophosphate; FdUDP, 5-fluoro-2′-*deoxy*uridine 5′-diphosphate; FdUTP, 5-fluoro-2′-*deoxy*uridine 5′-triphosphate; dUMP, 2′-*deoxy*urdine 5′-monophosphate; dUDP, 2′-*deoxy*urdine 5′-diphosphate; dUTP, 2′-*deoxy*urdine 5′-triphosphate; dTMP, 2′-*deoxy*thymidine 5′-monophosphate; dTDP, 2′-*deoxy*thymidine 5′-diphosphate; dTTP, 2′-*deoxy*thymidine 5′-triphosphate; DHF, dihydrofolate; 5,10-MTHF, 5,10-methylene-tetrahydrofolate.

imbalance has deleterious consequences for DNA synthesis and repair, ultimately leading to cell death (Fig. 1) [2,3].

It would be useful to quantify the three intracellularly formed nucleotides that are responsible for the antineoplastic effect of 5-FU, in cells of patients that are treated with 5-FU or capecitabine. This would provide insight into the amount of 'activated drug' that has reached the site of action, information that could ultimately lead to more substantiated dosage regimens. In addition, the measurement of the intracellular nucleotide concentrations could provide insight into the mechanisms underlying treatment resistance. Given these benefits, it is not surprising that for other nucleoside analogs, measurements of intracellular monophosphates, diphosphates and triphosphates are increasingly being applied [4–8]. For 5-FU, such measurements would also be useful.

In the past decades, various analytical methods for the quantification of 5-FU nucleotides were described. However, most assays are not suitable for the measurement of cell samples obtained from patients that are treated with 5-FU or capecitabine. Some assays lack sensitivity, such as the methods reported for the quantification of FdUMP by HPLC-UV [9] or using capillary electrophoresis with UV-detection [10]. Also fluorine-19 nuclear magnetic resonance (^{19}F NMR) spectroscopy was not sufficiently sensitive for quantification of the individual 5-FU nucleotides in patients samples [11]. In addition, a substantial portion of the reported assays utilized a radioactive label [1,12], which makes these assays also useless for the measurement of samples of patients that are treated with unlabeled drug.

For a long time, competitive-binding assays, wherein quantification was based on the displacement of radiolabeled isotopes, were

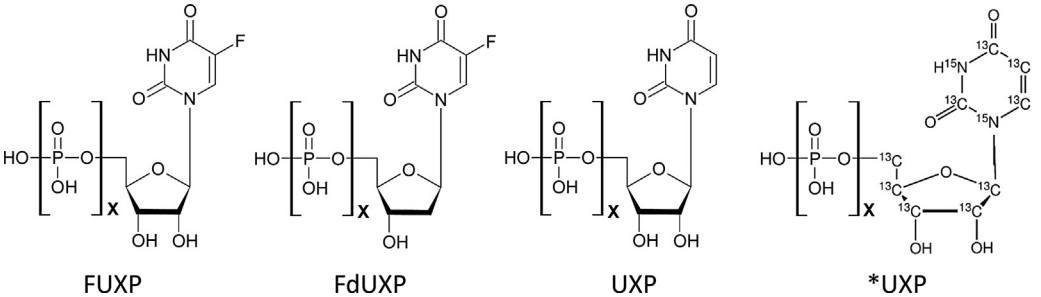

Fig. 2. Chemical structures of 5-fluorouridine-X-phosphate, 5-fluoro-2′-*deoxy*uridine-X-phosphate, uridine-X-phosphate and $^{13}C_9{}^{15}N_2$-labeled uridine-X-phosphate (tested as internal standard). X may be mono, di or tri.

the only option to measure FdUMP in patient samples [13–15]. However, these assays were quite laborious and not practical for clinical application. In 2009 Carli et al. described an LC–MS/MS assay for the quantification of FdUMP in cultured cell models [16]. This assay was much easier to apply and, if it is sensitive enough, it might also be used for the determination of FdUMP concentrations in patients samples.

To our knowledge, no LC–MS/MS assays have been reported for the quantification of FdUTP and FUTP. Therefore, our aim was to develop an LC–MS/MS assay for simultaneous quantification of the three 5-FU nucleotides that are responsible for the antineoplastic effect of 5-FU in peripheral blood mononuclear cells (PBMCs). PBMCs were used to reflect the intracellular metabolic activation because these cells can be easily obtained after administration of 5-FU or capecitabine.

In general, the development of an adequate assay for the determination of intracellular nucleotide concentrations remains a challenge. The main obstacles are: (1) the very low concentrations present in the cells of patients; (2) the extent of release from the cell matrix, which is difficult to assess; and (3) the presence of endogenous nucleotides, which are highly similar and may cause interference.

For the 5-FU nucleotides, the development of an appropriate assay proved even more difficult than for other nucleotide analogs because intracellular 5-FU nucleotide concentrations, especially those following capecitabine treatment, are much lower [17,18]. Maximization of the release from the cell matrix and minimization of endogenous interference were therefore critical factors in obtaining an appropriate assay. This paper describes how the obstacles mentioned above were overcome to develop a selective and sensitive assay for the quantification of the three nucleotides that are responsible for the anticancer effect of 5-FU.

2. Materials and methods

2.1. Chemicals

FUTP and FdUTP were purchased from Sierra Bioresearch (Tucson, AZ, USA). Because isotopically labeled FUTP and FdUTP were not commercially available, $^{13}C_9{}^{15}N_2$-labeled uridine 5′-triphosphate (*UTP, Fig. 2) was tested as a potential internal standard. This *UTP was obtained from Sigma–Aldrich (St. Louis, MO, USA), just like uridine, 5-fluorouridine and 5-fluoro-2′-*deoxy*uridine. UPLC–MS grade methanol, acetonitrile and water were obtained from Biosolve Ltd (Valkenswaard, The Netherlands). Ammonium acetate (purity 98%), glacial acetic acid, 25% ammonia solution, 50% sodium hydroxide solution and potassium dihydrogen phosphate (purity >99.5%) were purchased from Merck (Darmstadt, Germany). Tetrabutylammonium dihydrogen phosphate (purity >99%) was obtained from AppliChem (Darmstadt, Germany). The Ficoll-Paque plus density gradient medium used for the isolation of PBMCs was purchased from GE Healthcare. Phosphate buffered saline pH 7.4 (PBS) was from Sigma–Aldrich.

2.2. Preparation and quantification of the reference and internal standard solutions

Because only the nucleoside triphosphates were commercially available, the nucleoside monophosphates and diphosphates were obtained in-house by thermal degradation. To this end, separate aqueous solutions of FUTP, FdUTP and *UTP (as potential internal standard) were placed in a water bath at 90 °C. In this paper the suffix -XP is used to refer to the obtained mixtures of nucleoside monophosphates, diphosphates and triphosphates.

Thermal degradation of the nucleoside triphosphates into lower phosphates was monitored using the HPLC-UV system previously described for the analysis of emtricitabine and tenofovir with some minor adjustments [19]. The mobile phase consisted of 10 mM tetrabutylammonium dihydrogen phosphate with 70 mM potassium dihydrogen phosphate in water adjusted to pH 7 and 7% methanol (v/v), and it was delivered isocratically to a Synergi hydro-RP column (150 mm × 2.0 mm ID, 4 μm particles; Phenomenex, Torrance, CA, USA) with a flow rate of 0.25 mL/min. A volume of 10 μL was injected using the autosampler thermostated at 4 °C. Absorption was measured at 268 nm for FUXP and FdUXP and at 262 nm for *UXP.

For each solution, thermal degradation was continued until approximately equal amounts of nucleoside monophosphate, diphosphate and triphosphate were obtained. This was achieved after approximately 4 h at 90 °C. After the samples were thoroughly cooled on ice, the nucleotide concentrations of the FUXP, FdUXP and *UXP mixtures were determined using the above HPLC-UV system. 5-fluorouridine, 5-fluoro-2′-*deoxy*uridine and uridine were used as calibration standards based on the assumption that the nucleoside and the nucleotides have identical molar absorptions. To this end, three 5-fluorouridine, 5-fluoro-2′-*deoxy*uridine and uridine reference solutions were prepared from separate weighings. The peak areas obtained by injecting these reference solutions in duplicate were compared to those obtained by injecting the nucleotide solutions with unknown concentrations in triplicate. The FUXP and FdUXP mixtures were combined and diluted in water to obtain working solutions. The *UXP solution was diluted in water to obtain the internal standard working solution. All solutions were stored at −70 °C.

2.3. Sample preparation

Clinical samples were obtained by drawing 16 mL whole blood in lithium heparin tubes. Samples were processed immediately after blood collection, and processing was executed on ice. After centrifugation (10 min at 1500 × *g* at 4 °C), the buffy coat was collected and resuspended in cold phosphate buffered saline pH 7.4 (PBS) to a total volume of 6 mL. This diluted buffy coat was then carefully layered over 4 mL cold Ficoll-Paque plus density gradient, avoiding mixing of the layers. After centrifugation (20 min at 550 × *g* at 4 °C without brake), a two-layer system was formed. The PBMCs, forming a white interface between the upper and lower layers, were collected and washed with 35 mL cold PBS. After centrifugation (5 min at 1500 × *g* at 4 °C), the supernatant was thoroughly removed. The cell pellet was resuspended in 70 μL PBS, resulting in a homogeneous cell suspension with a total volume of approximately 100 μL. A 30 μL aliquot of this cell suspension was 10 times diluted with PBS and then used to perform a cell count using a hematology analyzer (Cell-Dyn Sapphire; Abbott Diagnostics, Abbott Park, IL, USA). A 60 μL aliquot of the cell suspension was used for determination of the 5-FU nucleotide concentrations. To this end, cells were lysed by the addition of 100 μL methanol, followed by extensive vortex mixing for 1 min. After centrifugation (5 min at 3000 × *g* at 4 °C), the supernatant (PBMC lysate) was collected and stored at −70 °C until analysis.

Directly prior to the LC–MS/MS analysis, after vortex mixing, a 100 μL sample was processed by the addition of 5 μL internal standard working solution. Then, the samples were again vortex mixed, centrifuged (5 min at 3000 × *g* at 4 °C) and transferred to autosampler vials.

2.4. Preparation of CALs and QCs

For the preparation of blanks, a blank human leukocyte buffy coat from 500 mL whole blood (Sanquin, Amsterdam, The

Netherlands) was diluted to 500 mL with PBS. This diluted buffy coat was layered over cold Ficoll-Paque plus density gradient and further treated as described in Section 2.3. The thus obtained blank PBMC suspension was diluted with PBS to 42×10^6 PBMCs/mL. This was previously reported as the average cell concentration after processing 15 mL whole blood [20]. Blank PBMC suspensions were lysed by the addition of methanol, wherein the volume ratio of 60:100 was maintained. After centrifugation (5 min at $3000 \times g$ at 4 °C), the supernatant (blank PBMC lysate) was stored at −70 °C.

Directly prior to the LC–MS/MS analysis, six non-zero calibration standards (CALs) with FUTP concentrations of 0.488, 0.974, 1.53, 1.99, 9.74 and 19.9 nM, FdUTP concentrations of 1.66, 3.31, 5.22, 6.77, 33.1 and 67.7 nM, and FdUMP concentrations of 0.748, 1.50, 2.36, 3.07, 15.0 and 30.7 nM were prepared by adding 5 μL of the appropriate working solution to 95 μL blank PBMC lysate. In the same manner, quality control samples (QCs) were prepared at mid-level (4.98 nM for FUTP, 16.9 nM for FdUTP and 7.66 nM for FdUMP). The CALs and QCs were further processed as described in Section 2.3.

2.5. LC–MS/MS system

The LC–MS/MS analyses were performed on an Acquity ultra performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA, USA) coupled to a QTrap 5500 mass spectrometer (AB Sciex, Framingham, MA, USA). Samples were kept at 4 °C in the autosampler tray, and a sample volume of 10 μL was loaded onto a Biobasic AX column (50 mm × 2.1 mm ID, particle size 5 μm) preceded by a 10 mm drop-in guard cartridge (both Thermo Scientific). The column oven was maintained at 40 °C. Mobile phase A consisted of a mixture of 10 mM NH_4Ac in ACN/H_2O (30:70, v/v) adjusted to pH 6.0, and mobile phase B consisted of 1 mM NH_4Ac in ACN/H_2O (30:70, v/v) adjusted to pH 10.5. The eluent pH was increased, and the NH_4Ac concentration was decreased in mobile phase B relative to mobile phase A to obtain a good separation of the nucleoside monophosphates, diphosphates and triphosphates. The mobile phases were delivered at a flow rate of 0.25 mL/min with the following stepwise gradient: initially 10% B; 0.1–1.6 min: 60% B; 1.65–2.1 min: 80% B; 2.11–5.0 min: 100% B; 5.1–6.9 min: 10% B with an increased flow rate of 0.50 mL/min; after which the flow returned to the initial conditions. The total run time was 7 min. A switching valve was used to direct the flow to waste during the first 1.5 min and after 6.9 min of the run.

The QTrap 5500 mass spectrometer was equipped with an electrospray ionization probe and was operated in the negative ion mode. The source settings and compound-dependent scan conditions were optimized through continuous infusion and flow injection analysis (FIA). The ionspray voltage was kept at −4500 V at a temperature of 700 °C. The curtain gas (N_2) and gas 1 and 2 (zero air) were set at 40 arbitrary units, and the collision-activated dissociation gas (N_2) was set at 11 arbitrary units. The entrance potential was −10 V. Scans of 50 ms were performed for each compound at unit resolution. After optimization, several mass transitions were compared for each analyte, and the mass transitions that resulted in the largest signal to noise ratios were selected (Table 1). Data were acquired and processed using Analyst (version 1.5.2) software (AB Sciex).

3. Results and discussion

3.1. Development of the assay

3.1.1. Maximization of nucleotide release from the cell

Accurate determination of intracellular nucleotide concentrations requires an efficient extraction from cells. However, evaluation of the extraction recovery is not simple. The intracellular situation cannot be simulated in a spiking experiment because it would not properly reflect cell lysis and extraction from the cell interior. To still be able to select the most suitable method for PBMC lysis and nucleotide extraction, we performed an experiment with a compound that is naturally present within cells: uridine 5′-triphosphate (UTP). Because the chemical structure of UTP is very similar to that of FUTP (Fig. 2), the UTP yields were considered a useful representative for FUTP liberation from the cells.

To compare extraction methods, 60 μL PBMC aliquots originating from a single buffy coat were used. Because these aliquots originated from the same buffy coat, their nominal UTP content was presumed to be constant. This provided the means to compare the *relative* UTP recoveries obtained with different extraction methods. Based on the methods for PBMC lysis and nucleotide extraction found in the literature, different approaches were evaluated. Each method was executed five times. Four extraction solvents were tested: methanol, ethanol, acetonitrile and 1.35 M perchloric acid ($HClO_4$). Three different amounts of extraction solvent were compared: 60, 100 and 140 μL. Except for perchloric acid, for which only a volume of 60 μL was tested because the perchloric acid treatment required further dilution for neutralization prior to LC–MS/MS analysis (by the addition of 25 μL 1 M ammonium acetate and 10 μL 10% ammonia). Finally, three different extraction procedures were evaluated. *Procedure 1*: Addition of extraction solvent to the PBMC suspension, followed by extensive vortex mixing and direct centrifugation, after which the supernatant was collected and stored at −70 °C. *Procedure 2*: Addition of extraction solvent to the PBMC suspension, followed by extensive vortex mixing, after which the entire sample (still containing cell debris) was stored at −70 °C. Thus, centrifugation was suspended until after thawing. *Procedure 3*: The PBMC suspension was directly stored (as a cell pellet) at −70 °C. Just prior to LC–MS/MS analysis, the sample was thawed, and extraction solvent was added, followed by extensive vortex mixing, centrifugation and collection of the supernatant. Evaporation of the extraction solvent to concentrate the extracts was not possible due to the instability of the analytes.

The UTP extraction potential of the extraction methods was evaluated using the described LC–MS/MS method. To this end, the method was extended with a mass transition for UTP (m/z 483 → m/z 159). In addition to a different UTP recovery, the various extraction methods could also cause a different matrix effect by the extraction of diverse other compounds from the cell matrix. However, because this experiment was intended to optimize the sensitivity of the 5-FU nucleotides assay presented in this paper,

Table 1
Selected mass transitions and scan conditions.

Compound	Parent ion (m/z)	Product ion (m/z)	Declustering potential (V)	Collision energy (V)	Collision cell exit potential (V)
FUTP	500.9	158.9	−95	−42	−13
FdUTP	484.8	256.8	−145	−44	−21
FdUDP	404.8	275.0	−105	−28	−9
FdUMP	325.0	128.9	−85	−26	−11
*UTP	493.8	158.9	−165	−44	−23
*UMP	334.1	117.2	−120	−34	−9

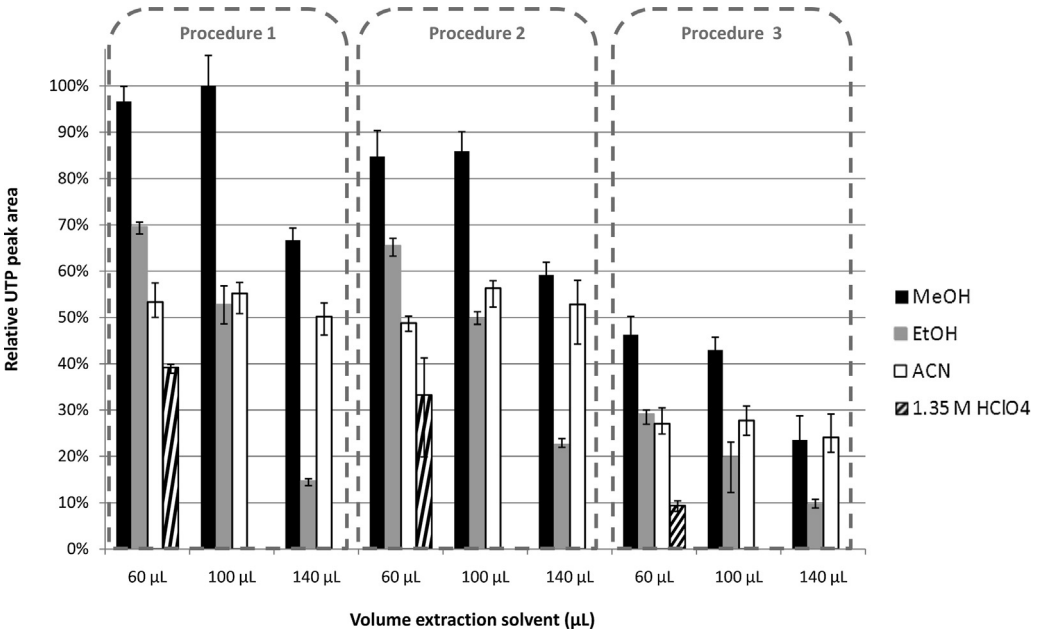

Fig. 3. Relative UTP peak areas obtained with different extraction methods. The largest peak area (defined as 100%) was obtained after extraction with 100 µL methanol following procedure 1. Each method was executed five times. The error bars represent the range between the highest and lowest observation.

we were interested in the extraction method with the most appropriate *resultant* of both matrix effect and recovery. Therefore, a pragmatic approach was followed: we searched for the extraction method that resulted in the highest UTP peak areas when analyzing the final extract.

The relative UTP peak areas obtained with the different extraction procedures are depicted in Fig. 3. The largest UTP peak areas were observed after extraction with 100 µL methanol according to procedure 1. The smallest UTP peak areas were obtained after extraction procedure 3. This extraction procedure was included in the comparison because direct freezing of the cell pellet would cause cell lysis, as PBMCs are not resistant to freezing. However, considering the poor results, this approach is probably detrimental with regard to the stability of UTP, which might be explained by the fact that enzymes were not inactivated by an extraction solvent before storage at −70 °C.

Because extraction procedure 1 with 100 µL methanol gave the most favorable combination of matrix effect and recovery, this extraction procedure was selected for the 5-FU nucleotides assay. Maximization of the nucleotide release from the cell was important to obtain maximum assay sensitivity. Furthermore, reproducibility of the recovery is important to obtain adequate assay precision. After extracting five blank PBMC samples with 100 µL methanol according to procedure 1, the coefficient of variation for the UTP peak areas was 6.5%, which was considered acceptable.

3.1.2. Minimization of endogenous interference

The obtained LC–MS/MS chromatograms show several endogenous interferences (Fig. 4). In the FUTP and FdUTP transitions, for instance, a peak was detected with a retention time of 3.02 min. Separation from this endogenous peak is essential to quantify FUTP and FdUTP and was achieved by the 80% eluent B step in the gradient. The pH of the eluents is critical to maintain separation. After 24 h, the pH of eluent B changed, and the separation deteriorated. Therefore, eluents were prepared freshly before each run. The small peaks observed in the 5-fluoro-2′-deoxyuridine 5′-diphosphate (FdUDP) and FdUMP transitions at 3.20 min are probably the consequence of in-source degradation of FdUTP. Therefore, chromatographic separation of the monophosphates, diphosphates and triphosphates is a necessity to prevent biased quantitation of the analytes. To examine if this chromatographic separation was achieved, the FdUDP mass transition was also monitored, even if FdUDP was not active.

3.1.3. Considerations regarding matrix effects and the use of internal standards

Matrix effects are relevant in LC–MS/MS because co-eluting compounds can interfere during the ionization process. The number of PBMCs varies per sample and may influence the matrix effect [21], so the matrix effect was studied for different cell concentrations. Clinical samples derived from 16 mL whole blood using the described isolation method were found to contain $10–200 \times 10^6$ PBMCs/mL. Therefore cell suspensions containing 5, 10, 25, 50, 100, 150, 200, 250, 300 and 350×10^6 PBMCs/mL were prepared in PBS using PBMCs freshly isolated from a single donor buffy coat. Sixty microliter aliquots of these cell suspensions were lysed with 100 µL methanol. After centrifugation, the PBMC lysate was used to prepare QCs at mid-level (FUTP 4.98 nM, FdUTP 16.9 nM and FdUMP 7.66 nM). Each PBMC concentration was processed and analyzed in triplicate. The matrix effect was evaluated by determination of the matrix factor at each PBMC concentration. To this end, the mean peak area found for samples with a particular PBMC concentration was divided by the mean peak area found for samples with the lowest PBMC concentration (5×10^6 PBMCs/mL) containing very little cell matrix.

In Fig. 5A the matrix factor for each analyte is displayed for different cell concentrations. The matrix factors for FUTP and FdUTP were independent of the number of PBMCs in the sample. In contrast, the matrix factor for FdUMP was strongly dependent on the number of PBMCs and decreased with increasing cell concentrations.

The matrix effects at different cell concentrations were also examined for the potential internal standards (Fig. 5B). Because isotopically labeled FUTP and FdUTP were not commercially available, $^{13}C_9{}^{15}N_2$-labeled uridine 5′-triphosphate (*UTP) was tested as a potential internal standard. However, the *UTP peak areas were found to increase dramatically with the number of cells. This was a consequence of an endogenous interference at the retention time of *UTP. In the calibration samples, with 42×10^6 PBMCs/mL, this endogenous interference caused a small peak whose

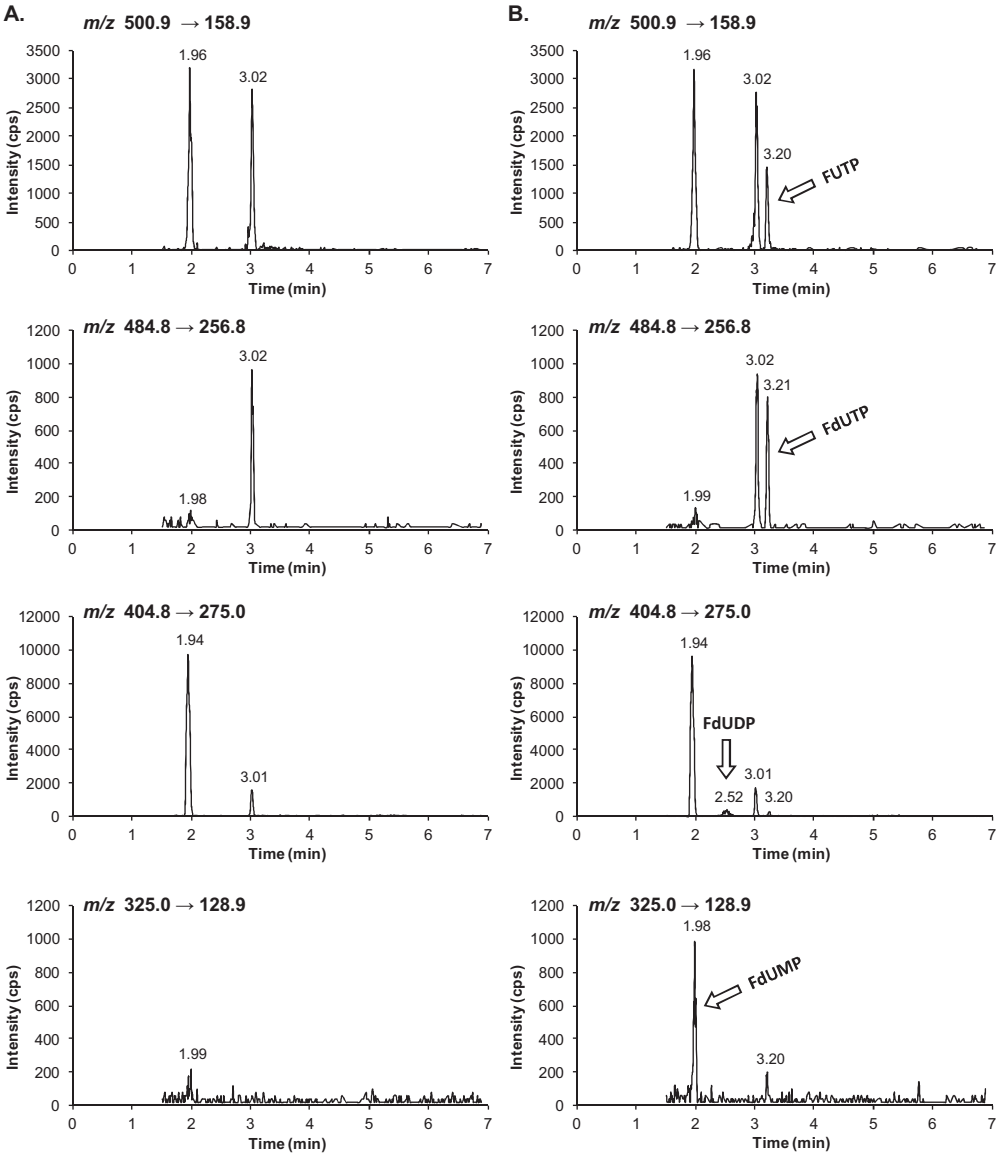

Fig. 4. Representative LC–MS/MS chromatograms for blank PBMC lysate (A) and PBMC lysate spiked with FUXP and FdUXP at the lower limit of quantification (B).

contribution was negligible, based on the signal-to-noise ratio. However, at higher cell concentrations, the endogenous contribution increased and was no longer insignificant. For this reason, *UTP was not suitable for use as an internal standard in this assay.

Despite an extensive search, we did not find another suitable internal standard that eluted near or at the same retention time as FUTP and FdUTP and was not affected by interference. However, because the matrix effects for FUTP and FdUTP remained constant for different cell numbers, the use of an internal standard was not necessarily required for quantitative determination of these analytes. We therefore decided not to use an internal standard for the quantification of FUTP and FdUTP.

For FdUMP on the other hand, the matrix factor was dependent on the amount of cells in the sample. Correction by means of an internal standard was therefore necessary for FdUMP. Because no endogenous interference was observed for *UMP, this compound was selected as the internal standard. The *UMP areas slightly decreased with increasing cell concentrations (Fig. 5B). In Fig. 5C, the relative matrix factors, corrected for the potential internal standards, are depicted. Although the relative matrix factor for FdUMP still decreased at higher cell amounts, the internal standard in a certain extent corrected for the decrease in the signal with the increase of the number of cells. We therefore decided to use *UMP as the internal standard for the quantification of FdUMP.

To further optimize the accuracy of the FdUMP quantification, it is possible to correct for the remaining matrix effect based on the number of PBMCs measured in a sample. To this end, for each PBMC concentration, a correction factor was determined by dividing the nominal FdUMP concentration by the measured concentration (Table 2). To correct for the remaining matrix effect, the measured FdUMP concentration in a sample should be multiplied by the correction factor associated with that particular cell concentration.

3.2. Validation of the assay

3.2.1. Linearity

Six non-zero calibration standards with the concentrations mentioned in Section 2.4 were prepared and analyzed in duplicate in three separate analytical runs. For FUTP and FdUTP, calibration curves were constructed using linear regression of the analyte peak area with a $1/x$ weighting factor (where x = concentration). For FdUMP, the analyte-internal standard peak area ratio was used. For all analytes, the lower limit of quantification (LLQ) was set at the

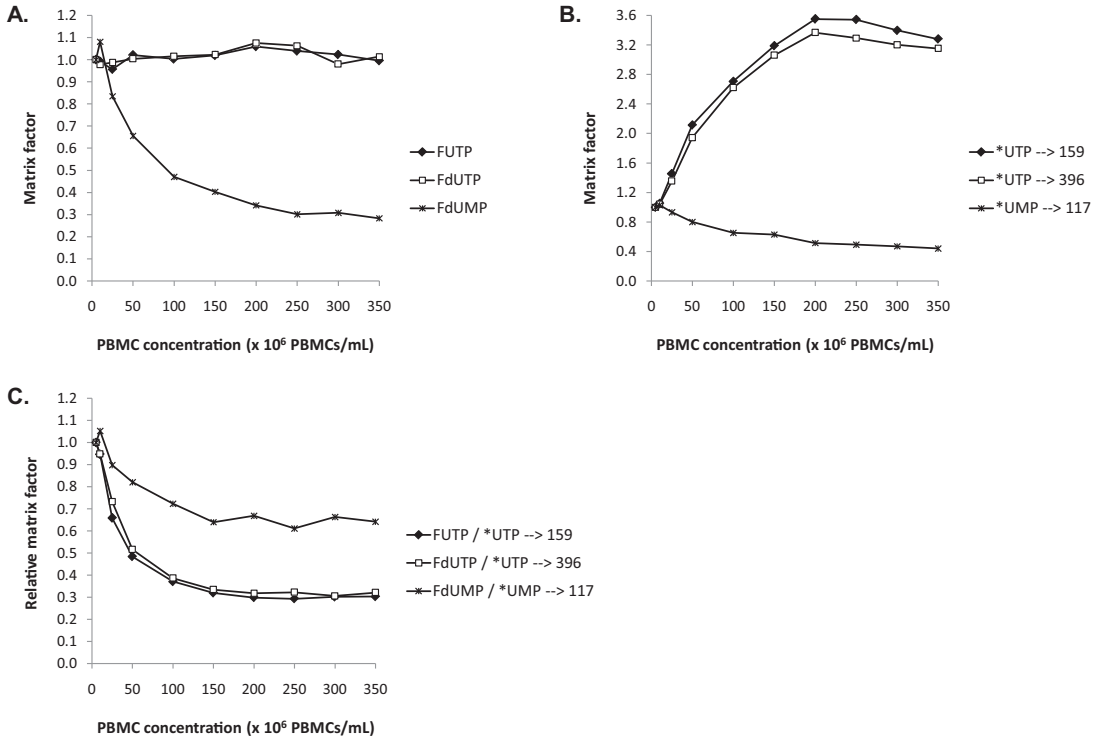

Fig. 5. Matrix factors for the analytes (A) and potential internal standards (B) and the relative matrix factors corrected for the internal standards (C) plotted for samples with different PBMC concentrations spiked at QC-mid-level.

lowest calibration standard. Linearity was evaluated based on the back-calculated concentrations of the calibration standards. Deviations from the nominal concentrations should be within ±20% at the LLQ and within ±15% at other concentrations. No more than 25% of the calibration standards were allowed to be rejected from the calibration curves.

The assay was linear over the validated concentration ranges in PBMC lysate from 0.488 to 19.9 nM for FUTP, from 1.66 to 67.7 nM for FdUTP and from 0.748 to 30.7 nM for FdUMP. The mean back-calculated concentrations did not deviate more than −3.3% and +4.4% from the nominal concentrations for all compounds at all levels. The coefficients of variation (CVs) were less than 10.5%, and the correlation coefficients (r) were at least 0.9948.

3.2.2. Accuracy and precision

Validation samples at mid-level (FUTP 4.98 nM, FdUTP 16.9 nM and FdUMP 7.66 nM) were analyzed in five replicates in three analytical runs. For each compound, the intra- and inter-assay accuracy and precision values were assessed. The accuracies (both intra- and inter-assay) were between −2.2 and 7.0% deviation, and the precision values were lower than 4.9% CV for all analytes. The dilution integrity was demonstrated by diluting validation samples above the upper limit of quantification (FUTP 99.6 nM, FdUTP 339 nM and FdUMP 153 nM) with blank PBMC lysate. A 50 μL aliquot of validation sample was diluted with 950 μL blank PBMC lysate in five replicates.

3.2.3. Selectivity

To determine the selectivity of the method, PBMCs were isolated from the blood of six different individuals who did not use 5-FU or capecitabine. The obtained blank samples were checked for interferences. No interferences were observed at the retention times of the analytes or internal standard.

3.2.4. Carry over

Carry over was determined by injecting a blank sample after the highest calibration standard and was less than 19.7% of the peak area of an LLQ sample for all analytes. Carry-over of the internal

Table 2
To correct for the relative matrix effect associated with a particular PBMC concentration, the measured FdUMP concentration in a sample should be multiplied by the experimentally determined correction factor.

PBMC concentration ($\times 10^6$ PBMCs/mL)	Nominal FdUMP concentration (nM)	Measured FdUMP concentration[a] (nM)	Correction factor (=nominal/measured FdUMP concentration)
5	7.66	8.89	0.86
10	7.66	9.35	0.82
25	7.66	7.97	0.96
50	7.66	7.27	1.05
100	7.66	6.41	1.20
150	7.66	5.64	1.36
200	7.66	5.92	1.30
250	7.66	5.40	1.42
300	7.66	5.86	1.31
350	7.66	5.67	1.35

[a] For each PBMC concentration, QC mid samples were processed and analyzed in triplicate. The measured FdUMP concentration is the mean of the three measurements.

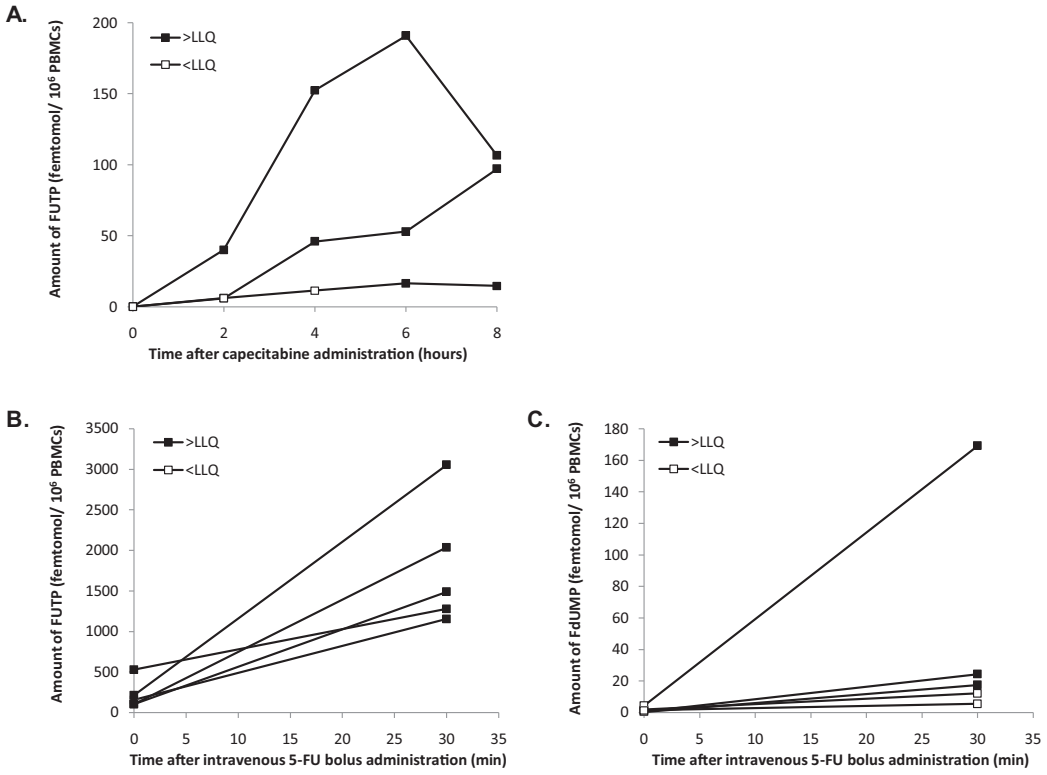

Fig. 6. FUTP amounts per 10^6 PBMCs determined for 3 patients after a single oral capecitabine dose of 850 mg/m^2 (A); and FUTP and FdUMP amounts per 10^6 PBMCs determined for 5 patients after an intravenous 5-FU bolus of 400 mg/m^2 in 30 min (B and C). Concentrations within the range of the assay are indicated by solid squares, and concentrations below the LLQ are represented by open squares.

standard was less than 3.7% of the internal standard peak area of a spiked sample.

3.2.5. Stability

Stability of the stock solutions was assessed after one year of storage at −70 °C. To this end, the nucleotide stock solutions were re-quantified on the basis of freshly prepared nucleoside reference standards, using the HPLC-UV system described in section 2.2. After one year of storage, the concentrations of the stock solutions did not deviate more than ±2.7% from the concentrations determined at $t = 0$.

Stability in PBMC lysate was evaluated using QC mid samples (FUTP 4.98 nM, FdUTP 16.9 nM and FdUMP 7.66 nM), which were analyzed after 3 h storage in an ice water bath, after 24 h in the autosampler at 4 °C and after one month storage at −70 °C. After storage, the QC samples were analyzed against a calibration curve obtained from freshly spiked calibration standards. The obtained concentrations did not deviate more than ±14.0% from the nominal value, indicating acceptable stability.

3.3. Clinical application of the assay

The described assay was applied for the determination of the 5-FU nucleotide concentrations in PBMC samples obtained from three patients treated with capecitabine. These patients participated in a phase I/II study in advanced gastric cancer. This study was approved by the Medical Ethics Committee of our Institute, and all patients provided written informed consent before enrollment. Patients received capecitabine at 850 mg/m^2 BID for fourteen days, every three weeks. Capecitabine tablets were taken with water within 30 min after a meal. Blood samples were collected on day one of the first treatment cycle, just before oral administration of capecitabine (pre-dose) and 2, 4, 6 and 8 h after oral capecitabine administration. Sampling times were chosen considering the plasma pharmacokinetics of capecitabine. When capecitabine is ingested with food, the maximal capecitabine and 5-FU plasma concentrations are reached after approximately 2 h. The terminal elimination half-lives of capecitabine and its 5-FU metabolite are less than 1 h [22]. After collection, blood samples were immediately placed on ice, and PBMCs were isolated and processed within 30 min.

In the resulting PBMC lysates obtained from patients treated with capecitabine, FUTP concentrations up to 1.84 nM were measured. The analytical results, expressed as nM in PBMC lysate, were multiplied by the lysate sample volume to obtain the absolute 5-FU nucleotide amounts in a sample. These amounts were then divided by the number of cells present in the sample to obtain the 5-FU nucleotide amounts per 10^6 PBMCs. The determined FUTP amounts per 10^6 PBMCs are presented in Fig. 6A and show considerable variability. The FdUTP and FdUMP concentrations in these patient samples were below the LLQ of the assay. This finding was not surprising because deoxyribonucleotide concentrations in cells are several orders of magnitude lower than ribonucleotide concentrations [23,24]. This makes sense given that deoxyribonucleotides are intended for incorporation into DNA, whereas ribonucleotides are intended for incorporation into RNA, and the DNA content of a cell is lower than the total RNA content [25,26].

Because potentially higher intracellular 5-FU nucleotide levels were expected after *intravenous* administration of 5-FU, we also collected blood samples from five patients receiving 5-FU intravenously. Patients received a modified FOLFOX6 regimen consisting of sequentially: oxaliplatin (85 mg/m^2 as a 2 h infusion), leucovorin (400 mg/m^2 as a 30 min infusion), a 5-FU bolus (400 mg/m^2 in 30 min), and a continuous 5-FU infusion (2400 mg/m^2 as a 44 h infusion), in the first 48 h of a 14-day treatment cycle. Blood samples were collected on day one of the second, third or fourth treatment cycle, pre-dose and 30 min after the end of the 5-FU bolus.

In the resulting PBMC lysates obtained after intravenous 5-FU, FUTP concentrations up to 178 nM were measured. In these samples, FdUMP was measured in concentrations up to 3.39 nM. Only the free FdUMP fraction is measured here because the FdUMP portion that is covalently bound to the enzyme thymidylate synthase precipitates during the extraction step with methanol [12,27]. The FUTP and FdUMP amounts per 10^6 PBMCs are presented in Fig. 6B and C, respectively. The FdUTP concentrations in the samples obtained after intravenous administration of 5-FU were still below the LLQ of the assay.

4. Conclusion

A sensitive LC–MS/MS method for the simultaneous quantification of FUTP, FdUTP and FdUMP in PBMC lysate was developed. The best method for cell lysis and nucleotide extraction was carefully selected, and the effect of different cell numbers was examined. The assay can be applied for the measurement of the intracellular FUTP concentrations in patient samples obtained after intravenous administration of 5-FU as well as after oral administration of its prodrug capecitabine. More sensitivity may be needed for the measurement of FdUTP in patient samples. FdUMP was only measurable with this assay after intravenous administration of 5-FU. This is the first report in the literature where 5-FU nucleotide concentrations could be measured directly in cells from patients treated with 5-FU or capecitabine without using a radioactive label. The clinical implications of these intracellular findings will become apparent after sampling more patients and will be discussed in a future paper.

References

[1] G. Weckbecker, Biochemical pharmacology and analysis of fluoropyrimidines alone and in combination with modulators, Pharmacol. Ther. 50 (1991) 367–424.

[2] J. Grem, Mechanisms of action and modulation of fluorouracil, Semin. Radiat. Oncol. 7 (1997) 249–259.

[3] P. Álvarez, J.A. Marchal, H. Boulaiz, E. Carrillo, C. Vélez, F. Rodríguez-Serrano, C. Melguizo, J. Prados, R. Madeddu, A. Aranega, 5-Fluorouracil derivatives: a patent review, Expert Opin. Ther. Pat. 22 (2012) 107–123.

[4] C. Bazzoli, V. Jullien, C. Le Tiec, E. Rey, F. Mentré, A.-M. Taburet, Intracellular pharmacokinetics of antiretroviral drugs in HIV-infected patients, and their correlation with drug action, Clin. Pharmacokinet. 49 (2010) 17–45.

[5] R.S. Jansen, H. Rosing, J.H.M. Schellens, J.H. Beijnen, Mass spectrometry in the quantitative analysis of therapeutic intracellular nucleotide analogs, Mass Spectrom. Rev. 30 (2011) 321–343.

[6] E.J.B. Derissen, M.J.X. Hillebrand, H. Rosing, H.M.M.B. Otten, E. Laille, J.H.M. Schellens, J.H. Beijnen, Quantitative determination of azacitidine triphosphate in peripheral blood mononuclear cells using liquid chromatography coupled with high-resolution mass spectrometry, J. Pharm. Biomed. Anal. 90 (2014) 7–14.

[7] R.S. Jansen, H. Rosing, J.H.M. Schellens, J.H. Beijnen, Simultaneous quantification of 2′,2′-difluorodeoxycytidine and 2′,2′-difluorodeoxyuridine nucleosides and nucleotides in white blood cells using porous graphitic carbon chromatography coupled with tandem mass spectrometry, Rapid Commun. Mass Spectrom. 23 (2009) 3040–3050.

[8] C. Crauste, I. Lefebvre, M. Hovaneissian, J.Y. Puy, B. Roy, S. Peyrottes, S. Cohen, J. Guitton, C. Dumontet, C. Perigaud, Development of a sensitive and selective LC/MS/MS method for the simultaneous determination of intracellular 1-beta-D-arabinofuranosylcytosine triphosphate (araCTP), cytidine triphosphate (CTP) and deoxycytidine triphosphate (dCTP) in a human follicular lymphoma cell line, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 1417–1425.

[9] M. Barberi-Heyob, J.L. Merlin, B. Webér, Determination of 5-fluorouracil and its main metabolites in plasma by high-performance liquid chromatography, J. Chromatogr. 573 (1992) 247–252.

[10] A. Procházková, S. Liu, H. Friess, S. Aebi, W. Thormann, Determination of 5-fluorouracil and 5-fluoro-2′-deoxyuridine-5′-monophosphate in pancreatic cancer cell line and other biological materials using capillary electrophoresis, J. Chromatogr. A.916 (2001) 215–224.

[11] N.W. Lutz, W.E. Hull, Assignment and pH dependence of the ^{19}F-NMR resonances from the fluorouracil anabolites involved in fluoropyrimidine chemotherapy, NMR Biomed. 12 (1999) 237–248.

[12] A. Pogolotti, P. Nolan, D. Santi, Methods for the complete analysis of 5-fluorouracil metabolites in cell extracts, Anal. Biochem. 117 (1981) 178–186.

[13] G.J. Peters, J. Lankelma, R.M. Kok, P. Noordhuis, C.J. van Groeningen, C.L. van der Wilt, S. Meyer, H.M. Pinedo, Prolonged retention of high concentrations of 5-fluorouracil in human and murine tumors as compared with plasma, Cancer Chemother. Pharmacol. 31 (1993) 269–276.

[14] C.L. Van der Wilt, H.M. Pinedo, K. Smid, G.J. Peters, Elevation of thymidylate synthase following 5-fluorouracil treatment is prevented by the addition of leucovorin in murine colon tumors, Cancer Res. 52 (1992) 4922–4928.

[15] G.J. Peters, E. Laurensse, H.W. Steinbusch, J. De Vente, K. Smid, C.L. Van der Wilt, H.M. Pinedo, Development, characterization and application of an antibody against 5-fluoro-2′-deoxyuridine-5′-monophosphate, the active metabolite of 5-fluorouracil, Anticancer Res. 13 (1993) 835–839.

[16] D. Carli, M. Honorat, S. Cohen, M. Megherbi, B. Vignal, C. Dumontet, L. Payen, J. Guitton, Simultaneous quantification of, 5-FU 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP and TMP in cultured cell models by LC–MS/MS, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 2937–2944.

[17] L. Cattel, M. Airoldi, L. Delprino, R. Passera, P. Milla, F. Pedani, Pharmacokinetic evaluation of gemcitabine and 2′,2′-difluorodeoxycytidine-5′-triphosphate after prolonged infusion in patients affected by different solid tumors, Ann. Oncol. 17 (Suppl 5) (2006), v142-7.

[18] T. Yamauchi, E. Negoro, S. Kishi, K. Takagi, A. Yoshida, Y. Urasaki, H. Iwasaki, T. Ueda, Intracellular cytarabine triphosphate production correlates to deoxycytidine kinase/cytosolic 5′-nucleotidase II expression ratio in primary acute myeloid leukemia cells, Biochem. Pharmacol. 77 (2009) 1780–1786.

[19] R.S. Jansen, H. Rosing, W. Kromdijk, R. ter Heine, J.H. Schellens, J.H. Beijnen, Simultaneous quantification of emtricitabine and tenofovir nucleotides in peripheral blood mononuclear cells using weak anion-exchange liquid chromatography coupled with tandem mass spectrometry, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 621–627.

[20] S.A. Veltkamp, M.J.X. Hillebrand, H. Rosing, R.S. Jansen, E.R. Wickremsinhe, E.J. Perkins, J.H.M. Schellens, J.H. Beijnen, Quantitative analysis of gemcitabine triphosphate in human peripheral blood mononuclear cells using weak anion-exchange liquid chromatography coupled with tandem mass spectrometry, J. Mass Spectrom. 41 (2006) 1633–1642.

[21] F. Becher, A. Pruvost, J. Gale, P. Couerbe, C. Goujard, V. Boutet, E. Ezan, J. Grassi, H. Benech, A strategy for liquid chromatography/tandem mass spectrometric assays of intracellular drugs: application to the validation of the triphosphorylated anabolite of antiretrovirals in peripheral blood mononuclear cells, J. Mass Spectrom. 38 (2003) 879–890.

[22] B. Reigner, K. Blesch, E. Weidekamm, Clinical pharmacokinetics of capecitabine, Clin. Pharmacokinet. 40 (2001) 85–104.

[23] P. Chen, Z. Liu, S. Liu, Z. Xie, J. Aimiuwu, J. Pang, R. Klisovic, W. Blum, M.R. Grever, G. Marcucci, K.K. Chan, A LC–MS/MS method for the analysis of intracellular nucleoside triphosphate levels, Pharm. Res. 26 (2009) 1504–1515.

[24] S. Cohen, M. Megherbi, L.P. Jordheim, I. Lefebvre, C. Perigaud, C. Dumontet, J. Guitton, Simultaneous analysis of eight nucleoside triphosphates in cell lines by liquid chromatography coupled with tandem mass spectrometry, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 877 (2009) 3831–3840.

[25] S. Cohen, L.P. Jordheim, M. Megherbi, C. Dumontet, J. Guitton, Liquid chromatographic methods for the determination of endogenous nucleotides and nucleotide analogs used in cancer therapy: a review, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 1912–1928.

[26] G.M. Malacinski, Essentials of Molecular Biology, fourth ed., Jones & Bartlett Learning, 2005.

[27] W.L. Washtien, D.V. Santi, Assay of intracellular free and macromolecular-bound metabolites of 5-fluorodeoxyuridine and 5-fluorouracil, Cancer Res. 39 (1979) 3397–3404.