Study of intracellular anabolism of 5-fluorouracil and incorporation in nucleic acids based on an LC-HRMS method

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Abstract

5-Fluorouracil (5-FU) is an anticancer drug extensively used for different cancers. Intracellular metabolic activation leads to several nucleoside and nucleotide metabolites essential to exert its cytotoxic activity on multiple cellular targets such as enzymes, DNA and RNA. In this paper, we describe the development of a method based on liquid chromatography coupled with high resolution mass spectrometry suitable for the simultaneous determination of the ten anabolic metabolites (nucleoside, nucleotide and sugar nucleotide) of 5-FU. The chromatographic separation was optimized on a porous graphitic carbon column allowing the analysis of the metabolites of 5-FU as well as endogenous nucleotides. The detection was performed on an Orbitrap® tandem mass spectrometer. Linearity of the method was verified in intracellular content and in RNA extracts. The limit of detection was equal to 12 pg injected on column for nucleoside metabolites of 5-FU and 150 pg injected on column for mono- and tri-phosphate nucleotide metabolites. Matrix effect was evaluated in cellular contents, DNA and RNA extracts for nucleoside and nucleoside metabolites. The method was successfully applied to i) measure the proportion of each anabolic metabolite of 5-FU in cellular contents, ii) follow the consequence of inhibition of enzymes on the endogenous nucleotide pools, iii) study the incorporation of metabolites of 5-FU into RNA and DNA, and iv) to determine the incorporation rate of 5-FdUMP into 18 S and 28 S sub-units of rRNA.

Keywords:
5-Fluorouracil
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Nucleotide
RNA
DNA
Incorporation rate

1. Introduction

5-Fluorouracil (5-FU) is an anticancer drug which belongs to the group of antimetabolites. Its development began more than 60 years ago and 5-FU still remains extensively used throughout the world in monotherapy as well as in combination chemotherapy. 5-FU is either administered by intravenous infusion or by oral route using the prodrug capecitabine which is metabolized to 5-FU by three enzymatic steps. 5-FU is a treatment for different types of cancer including colorectal and other gastrointestinal cancers, breast cancer, and head and neck cancer. 5-FU exerts its cytotoxic action after enzymatic intracellular conversion to several nucleotide metabolites (Fig. 1). Numerous articles and reviews have been published on antitumor activity of 5-FU and here we only report briefly the main points [1,2]. Mechanism of action involved three active compounds of the drug: 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP), 5-fluorouridine-5′-triphosphate (5-FUTP) and 5-fluoro-2′-deoxyuridine-5′-triphosphate (5-FdUTP). 5-FdUMP binds to thymidylate synthase (TS), inhibiting the transformation of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP). The consequence is the imbalance of deoxynucleotide pool leading to DNA damage due to perturbation in synthesis and misrepairation. DNA damages are also due to 5-FdUTP incorporation into DNA causing strand breaks. 5-FUTP is incorporated into different RNA species, leading to abnormal RNA...
function and processing and perturbation of cell growth. Moreover, Samuelsson [3] demonstrated that 5-FU inhibits pseudouridine synthases (PS), enzymes which convert uridine (Urd) to pseudouridine (Pseudourd) in RNA. Thus, 5-FU exhibits several cellular targets related to the anabolism phase of the drug. In addition to these three active compounds, seven others are included in the anabolism phase of 5-FU: 5-fluorouridine (5-FUrd), 5-fluoro-2'-deoxyuridine (5-FdUrF), 5-fluorouridine-5'-monophosphate (5-FUMP), 5-fluorouridine-5'-diphosphate (5-FUDP), 5-fluoro-2'-deoxyuridine-5'-diphosphate (5-FdUDP), 5-FUDP-hexose (5-FUDP-Hex) and 5-FUDP-N-acetylhexosamine (5-FUDP-HexNAc) (Fig. 1) [4,5].

Several analytical methods reported the analysis of metabolites of 5-FU in biological matrix. While Procházková et al. [6] used capillary electrophoresis to quantify 5-FdUMP, most of other methods consist in liquid chromatography coupled with radioimmunoassay (RIA), diode-array detector (DAD) or mass spectrometry. Indeed, analysis of 5-FUrd and 5-FdUrd was reported in plasma [7–11] and in cell cultures [12–14]. Analysis of 5-FUMP and 5-FdUMP were described in serum [15], plasma [16], peripheral blood mononuclear cells (PBMC) [17], tissues [15] and cell cultures [12,13]. The diphosphorylated metabolites 5-FUDP and 5-FdUDP were studied in cell cultures [12] and the triphosphorylated forms 5-FUTP and 5-FdUTP were analyzed in plasma [16], PBMC [17] and cell cultures [12]. However, to the best of our knowledge, among all these methods, none allows the simultaneous analysis of the ten metabolites of the anabolism phase of 5-FU.

For the study of incorporation of metabolites of 5-FU into RNA and DNA, Benz et al. [18] and then Peters et al. [19] described a radiolabeled method applied after extraction and digestion of the nucleic acids. Keniry et al. [20] and el-Tahtawy et al. [21] reported the incorporation of metabolites of 5-FU into RNA using nuclear magnetic resonance (NMR) spectroscopy. A gas chromatography-mass spectrometry (GC-MS) method based on enzymatic degradation of 5-FUMP to 5-FU and derivatization with pentafluorobenzylbromide before analysis was also published [22–24]. In a study concerning the mechanisms for cytotoxicity of 5-FU, Pettersen et al. [25] used an LC-MS/MS method for the simultaneous analysis of 5-FUrd and 5-FdUrd in nucleic acids.

The aim of this work was to develop an assay based on LC-MS/HRMS, allowing the simultaneous determination of the ten nucleoside and nucleotide metabolites of the anabolism phase of 5-FU. In addition, we determined the main endogenous nucleosides and nucleotides of Urd to better illustrate the potential of this method to study i) the anabolic metabolites of 5-FU in cells, ii) the incorporation rate of 5-FU into DNA, RNA and subunit of ribosomal RNA, and iii) the consequence of TS and PS inhibition by 5-FU on endogenous nucleotide pools.

2. Material and methods

2.1. Chemicals and reagents

5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, Urd, 13C5-uridine (Urd13C5), uridine-15N2, 5'-monophosphate (UMP15N2), uridine-13C6,15N2, 5'-monophosphate (UMP13C6,15N2), uridine-13C6,15N2, 5'-triphosphate (UTP13C6,15N2), adenosine-5'-triphosphate (ATP), phosphodiesterase I from Crotalus adamanteus venom, alkaline phosphatase and benzamide were purchased from Sigma-Aldrich (St-Louis, USA). Deoxy-Nucleotide Monophosphate kinase (dNMP kinase) from bacteriophageT4 recombinant (E. Coli), 5-FdUMP and 5-FUTP were purchased at Jena Biosciences (Jena, Germany). 13C5-adenosine (Ad13C5) came from Eurisotop (Saint-Aubin, France). Nuclease S1 was from Promega (Madison, USA) and calf 12 intestine phosphatase from New England Biolabs™ (Evry, France). Pseudourd was from Santa Cruz Biotechnology (Heidelberg, Germany). PBS and trypsin-EDTA (0.05%) were purchased at Life technologies™ (ThermoFisher Scientific™, Les Ulis, France). Acetonitrile and methanol both of HPLC-grade, ammonia hydroxide aqueous solution (20%), acetic acid, TRIS hydrochloride, sodium chloride and magnesium chloride were obtained from Sigma-Aldrich™. Water filtered with an Elga Purelab (Flex system™, High Wycombe, United Kingdom) was used in all experiments.

2.2. Synthesis of 5-FUMP and pseudoUMP

5-FUMP was prepared by selective 5'-phosphorylation of 5-fluorouridine with POCl3 in triethylphosphate, followed by hydrolysis with triethylammonium bicarbonate (TEAB, 1 M, pH 7.5) [26–28]. Pseudouridine 5'-monophosphate was synthesized starting from Pseudourd, via a 5'-H-phosphonate approach [29]. The nucleotides were purified on DEAE-Sephadex A-25 (elution: gradient of TEAB pH 7.5 from 10 mM to 0.5 M), followed by chromatography on RP18 (elution: water to 50% methanol). The triethylammonium counter ions were exchanged to sodium by passing

![Fig. 1. Anabolism phase of 5-fluorouracil (5-FU). Enzymes involved in activation: ① uridine phosphorylase, ② thymidine phosphorylase, ③ orotate phosphoribosyltransferase, ④ thymidine kinase, ⑤ uridine kinase, ⑥ UMP-CMP kinase, ⑦ nucleoside diphosphate kinase, ⑧ ribonucleotide diphosphate reductase, ⑨ dUTP hydrolase, ⑩ UDP-glucose pyrophosphorylase, ⑪ UDP-N-acetylhexosamine pyrophosphorylase, and ⑫ thymidylate kinase.](image-url)
the 5-FdUDP and 5-FUDP nucleotide solution through a DOWEX-AG 50WX2-400 column (Fluka). Yields were 10%–19%.

5-FUMP, sodium salt. 1H NMR (400 MHz, D2O): δ 8.23 (d, JHH = 6.3 Hz, 1H, H6), 6.01 (dd, JHH = 5.2 Hz and JHE = 1.5 Hz, 1H, H1), 4.41 (t, JHH = 5.2 Hz, 1H, H2), 4.37 (t, JHH = 4.1 Hz, 1H, H3), 4.25–4.30 (m, 1H, H4), 3.91–4.10 (m, 2H, H5, H5’); 31P NMR (162 MHz, D2O) δ 3.38 (s); HRMS (ESI) m/z: [M−Na]− Calcd for C9H11FN2O9P 341.0186, found: 341.0190.

Pseudouridine 5’-monophosphate, sodium salt. 1H NMR (400 MHz, D2O): δ 7.76 (s, 1H, H6), 4.76 (d, JHH = 4.0 Hz, 1H, H1), 4.20–4.25 (m, 1H, H3), 4.12–4.16 (m, 1H, H2), 3.80–4.03 (m, 3H, H4, H5, H5’); 31P NMR (162 MHz, D2O) δ 3.90 (s); HRMS (ESI) m/z: [M−Na]− Calcd for C8H12N2O4P 323.0280, found: 323.0275.

2.3. Production of 5-FdUDP and 5-FUDP

The two nucleotide metabolites 5-FdUDP and 5-FUDP were produced by incubation of 50 µL of 5-FdUMP or 5-FUMP at 0.2 mM with dNMP kinase (5 µL containing 1000 units) for 30 min in a Tris-HCl buffer (pH 7.4; 100 mM) containing MgCl2 (10 mM) and ATP (2 mM). The final volume of the solution was 100 µL. Then 10 µL of incubation medium were added with 100 µL of a mixture of cold methanol/water (70:30, V/V) to stop the reaction. Samples were analyzed before the reaction and at 30 min.

2.4. LC-MS-HRMS instrumentation

Liquid chromatography analysis was performed on an Ultimate 3000 system (ThermoFisher Scientific™, Bremen, Germany) equipped with two ternary pumps. The LC system was coupled with a Q-Exactive Plus Orbitrap mass spectrometer (ThermoFisher Scientific™, Bremen, Germany). The separation of the compounds was carried out with a Hypercarb™ column (2.1 mm × 100 mm, 5 µm; ThermoFisher Scientific™, Bremen, Germany) and was thermostatted at 30 °C. The autosampler tray was maintained at 5 °C. A volume of 10 µL was injected. A stepwise gradient program with (A) NH4OH 0.25% adjusted to pH 10 with acetic acid, (B) water and (C) acetonitrile was performed. The electrospray source (ESI) operated alternatively in negative and positive modes. Spray voltage was set at 3 kV and 3.2 kV in negative and positive modes, respectively. The pressure of nitrogen sheath gas and auxiliary gas were maintained at 30 and 20 units (arbitrary units), respectively. The capillary temperature was 320 °C. A switching valve directed the eluate to waste during the first minute of the run and during reequilibration step. Mass spectrometer could operate in full scan mode (FS) and parallel reaction monitoring mode (PRM).

2.5. Samples preparation

2.5.1. Cell culture, exposure to 5-FU and isolation

HTC116 cells were grown in Dulbecco Minimum Essential Medium – GlutaMax (Invitrogen™, Villebon sur Yvette, France) supplemented with 10% fetal bovine serum (FBS). Cells were plated 48 h before 5-FU or 5-FdUrD exposure. HepaRG cells, purchased from Biopredic International™ (Rennes, France), were grown at a low density in Williams’ E medium supplemented with 10% FCS, 100 units/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine and 50 µM hydrocortisone hemisuccinate. After 2 weeks, the culture medium was supplemented with 1% DMSO and the cells were left to differentiate for 1 week (confluent DMSO-treated cells) [30].

Cells were exposed to 5-FU or 5-FdUrD for 24 h at 10 µM or 50 µM before cell isolation. For each experiment control samples consisting in non-treated cells were prepared. After exposure, cells were trypsinized after 2 washes with cold PBS. The cells were counted by adding 10 µL of trypan blue with 10 µL of cells, and then the mixture was introduced in automated cell counter (Countess™, Invitrogen, ThermoFisher, France). Cell pellet was washed once with cold PBS and then stored at −80 °C after removing the supernatant.

2.5.2. Extraction of intracellular metabolites

Intracellular content was extracted from cell pellet by lysis with a mixture of cold methanol/water (70:30, V/V) (Fig. 2). The following internal standards were added at 0.02 mM: Urd13C, UMP13C15N and UTP13C15N. Then samples were vigorously vortexed and centrifuged for 10 min at 13,000 g. Supernatant was evaporated to dryness under nitrogen at 37 °C. The residue was resuspended with 100 µL of water and then transferred to a vial for injection.

2.5.3. Extraction, hydrolysis and dephosphorylation of RNA

RNA extraction was performed using RNaseasy Mini Kit (Qiagen™, Les Ulis, France) after lysis of cell pellet with the lysis buffer provided by the kit. Quantity of RNA extracted was measured using a spectrophotometer NanoDrop 2000™ (Ozyme™, Saint-Cyr-L’École, France). Samples were then divided in aliquots containing a maximum of 3 µg of RNA before hydrolysis. RNA extracts was performed overnight at 37 °C by incubation with 270 units of Nuclease S1 using the supplied buffer. For analysis of RNA extracts under dephosphorylated form a dephosphorylation step was achieved by adding 5 U of calf intestine phosphatase in 100 mM Tris–HCl, 50 mM NaCl, 10 mM MgCl2 and 0.025% Triton X-100. Digested extracts were stored at −80 °C before analysis.

Before dephosphorylation, samples were spiked with 20 µL of UMP13C5N to be used as a standard for this step. Thus, UMP13C5N was dephosphorylated in Urd13C and UMP13C15N was followed during analysis in samples which underwent dephosphorylation.

A volume of 300 µL of pure methanol was added to RNA extracts. Then, 10 µL of the following internal standards at 0.02 mM were added: Urd13C, Ad13C and UMP13C15N. Then samples were vigorously vortexed and centrifuged for 10 min at 13,000 g. Supernatant was evaporated to dryness under nitrogen at 37 °C. The residue was resuspended with 100 µL of water and then transferred to a vial for injection.

2.5.4. Extraction, hydrolysis and dephosphorylation of DNA

DNA extraction was performed using QiAamp DNA Mini Kit™ (Qiagen™, Les Ulis, France) after lysis of cell pellet with the lysis buffer provided by the kit. Quantity of DNA extracted was measured using a spectrophotometer NanoDrop 2000™ (Ozyme™, Saint-Cyr-L’École, France). The procedure of digestion of DNA to deoxyribonucleoside was derived from a previous work published by Quinilvan and Gregory [31]. Briefly, DNA extracts were divided in aliquots containing 3 µg of DNA. Then 150 µL of digest mix was added. The composition of the digest mix was 250 U Benzonase™ nuclease, 300 mU phosphorydiesterase 1 (Sigma-Aldrich, Lyon, France) and 200 U alkaline phosphatase (New England Biolabs™, Eryv, France) to 5 mL Tris–HCl buffer (20 mM, pH 7.9) containing 100 mM NaCl and 20 mM MgCl2. DNA extracts were then incubated overnight at 37 °C. In the end, samples were stored at −80 °C until analysis.

To analyze samples representing a higher quantity of DNA, a supplementary step of concentration was performed. Briefly, five DNA digested extracts were pooled after resuspension with 300 µL of pure methanol. Then, 10 µL of Urd13C and Ad13C at 0.02 mM were
added as internal standard. The total volume was evaporated under nitrogen at 37°C and the residue was resuspended with 100 µL of water and transferred to a vial for injection. Finally, 20 µL was injected. Thus, this concentration step allowed the analysis of samples containing 10-fold higher DNA than the procedure described for RNA extracts.

2.5.5. 18 S and 28 S ribosomal RNA purification

Ribosomes were purified from HCT116 cells as previously described by Belin et al. [32] by sedimentation through sucrose cushion. Ribosomal RNAs (rRNAs) were purified using RNeasy Mini Kit as described above (Section 2.5.3). For 18 S and 28 S rRNAs purification, total rRNAs were separated by electrophoresis in a 1% agarose gel and the bands corresponding to 18 S and 28 S rRNA were visualized under UV fluorescence and cut out of the agarose gel. Each RNA was extracted from the agarose gel piece using the NucleoSpin RNA kit and NTC buffer (Macherey-Nagel, Hoerdt, France). Then, 18 S and 28 S rRNAs were quantified and digested and stored at −80°C as described in Section 2.5.3. The rest of preparation was identical to the one described above for DNA and RNA with the addition of 300 µL of pure methanol and 10 µL of Urd13C and Ad13C at 0.02 mM.

2.6. Method evaluation

2.6.1. Linearity of the method

To ensure the linearity of the analytical method for cellular content, samples containing increased intracellular contents of cells exposed to 50 µM 5-FU during 24 h were analyzed. Thus, lysates of HCT116 cells representing 0.5×10^6, 1×10^6, 2×10^6 and 3×10^6 cells were prepared. Linearity was verified by plotting the peak area ratio (metabolites of 5-FU/internal standard) as function of the cell number. Linearity was also tested by spiking intracellular extracts of 1×10^6 cells with increasing concentrations of 6 metabolites available in pure form. The range of the quantities injected was from 0.1 to 3.75 ng for 5-FUrd and 5-FdUrd, from 0.5 to 10 ng for 5-FUMP and 5-FdUMP and from 1 to 10 ng for 5-FUTP and 5-FdUTP.

To ensure the linearity of the analytical method for RNA extracts, samples containing increasing quantity of RNA extracted from cells exposed to 50 µM 5-FU during 24 h were analyzed. Thus, quantities of extracted RNA of 0.2, 0.5, 1 and 3 µg were hydrolyzed and dephosphorylated. Linearity was verified by plotting the peak area ratio (5-FUrd/internal standard) as function of the quantity of RNA.

2.6.2. Matrix effect

For cellular content, matrix effect of 5-FUrd, 5-FdUrd, 5-FUMP, 5-FdUMP, 5-FUTP and 5-FdUTP was studied by spiking non-treated cells extracts with 30 ng of each metabolite. Results were compared with the signal obtained by injecting on the chromatographic column the same quantity of the metabolites from a pure solution.

For nucleic acids, matrix effect was studied by spiking non-treated phosphorylated and dephosphorylated RNA with 5-FUMP and 5-FUrd, respectively and non-treated phosphorylated and dephosphorylated DNA with 5-FdUMP and 5-FdUrd, respectively. For the four metabolites, a quantity of 3 ng was added to DNA or RNA extracts of HCT116 cells. Samples were then prepared as explained previously. Results were compared with the signal obtained by injecting on the chromatographic column the same quantity of the metabolites from a pure solution. Experiments were performed three times. The ion suppression was also investigated by a post-column infusion system. Extract samples from non-treated DNA or RNA were injected with LC device, and post-column infusion of 5-FUrd, 5-FdUrd, 5-FUMP or 5-FdUMP was performed during the chromatographic run.

2.6.3. Determination of the limit of detection

Limits of detection (LOD) were determined for 5-FUrd, 5-FdUrd, 5-FUMP, 5-FdUMP, 5-FUTP and 5-FdUTP. For this work, we defined the LOD as the lowest injected quantity exhibited a coefficient of variation lower than 20% after analysis of three samples.

2.7. Applications

The study of the intracellular metabolites of the anabolism phase of 5-FU was performed on cell extracts after exposure to 5-FU or 5-FdUrd at 50 µM during 24 h. To observe the effect of exposure to 5-FU or 5-FdUrd on TS dUMP, dUDP, dUTP, TMP, TDP and TTP were quantified in these cell extracts. Incorporation of metabolites of 5-FU into DNA and RNA and determination of the incorporation rate into RNA were studied on RNA, DNA and ribosome extracts of HCT116 cells exposed to 5-FU or 5-FdUrd at 10 or 50 µM during 24 h.

The quantification of 5-FUrd, adenosine, guanosine, cytidine, Urd and pseudoUrd in RNA extracts was performed using
calibration curves. Concentrations of the lowest standard and the highest standard were equal to 0.03 and 5.00 μg/mL for Urd, adenosine, guanosine and cytidine, 1.67 and 250 ng/mL for pseudourd, and 0.21 and 31.25 ng/mL for 5-FUr. Urd13C was used as internal standard for 5-FUr, Urd, pseudourd and cytidine, and Ad13C was the internal standard of adenosine and guanosine. Calibration curves were constructed by plotting the ion abundance peak area ratio (5-FUr or endogenous nucleosides/internal standard) as function of metabolite concentration. Data were fitted by weighted (1/concentration) for least-squares regression, and standard curves were determined using linear regression analysis.

3. Results

3.1. Analytical method for the identification of metabolites of 5-FU

3.1.1. LC-MS-HRMS method

A liquid chromatography method was developed for the simultaneous determination of the ten nucleoside and nucleotide metabolites of the anabolism phase of 5-FU. Six metabolites (5-FUr, 5-FdUr, 5-FUMP, 5-FdUMP, 5-FUTP and 5-FdUTP) available as pure compounds were used for the development of the method. 5-FUMP was synthesized since this compound was not commercially available. The study of the chromatographic behavior and mass spectrometry characteristics of nucleoside and nucleotide metabolites was performed. The method was adapted from a previously validated assay for the quantification of endogenous nucleotides [33]. A stepwise gradient program with (A) 0.25% NH4OH adjusted to pH 10 with acetic acid, (B) water and (C) acetonitrile was performed (Table 1). The percentage of (A) was maintained to 10%, while the percentage of (C) was increased from 12% to 53%. The column was equilibrated with 10% of (A) and 90% of (B). Equilibration of the column was performed without acetonitrile to allow the retention of nucleosides on the Hypercarb® column. Metabolites of 5-FU were eluted between 4.5 and 6.1 min (Table 1 and Fig. 3).

On the Q-Exactive Plus mass spectrometer, full scan mode from m/z 100 to m/z 750 with a resolution of 70,000 was applied after positive and negative ionization. AGC target was programmed to 1 x 106 and the max IT was 100 ms. For all analytes, response after positive and negative ionization were compared. The ionization mode inducing the better signal to noise ratio was selected. Thus, negative ionization mode were selected for 5-FUr, 5-FdUr, 5-FUMP, 5-FdUMP, 5-FUTP and 5-FdUTP available as pure compounds. Thus, to test the ability of our method to detect all the metabolites, 5-FUDP-Hex and 5-FUDP-HexNaC were produced from cell lines, and 5-FUDP and 5-FdUDP came from an enzymatic reaction.

In order to produce diphosphorylated metabolites of 5-FU, 5-FUDP and 5-FUMP were incubated separately with dNMP kinase. Although the dNMP kinase exhibits a high selectivity for dNMP substrate, NMP substrate can also be metabolized in a limited extent [34]. Incubation of 5-FdUMP or 5-FUMP with dNMP kinase showed a decrease of both substrates over time. With 5-FdUMP, signal intensity increased at 4.5 min with m/z at 407.00514 in positive mode and 404.99058 in negative mode and corresponding to the chemical formula C8H13F3N3O11P2. The isotopic abundance was also in agreement (5 ppm) with this chemical formula. The signal intensity was about 50 times higher in negative mode than in positive mode. The fragmentation using PRM mode (Rs = 17,500) exhibited main fragments at m/z 78.95904, 158.92541, 96.96953 and 129.01057 (Table 2). The fragment at m/z 129.01057 corresponds to the nucleobase 5-FU. Other main fragments corresponded to phosphorylated moiety. All these data strongly suggested that 5-FUDP was produced by the enzymatic reaction. With 5-FUMP, signal intensity increased at 4.5 min only in negative mode with m/z at 420.98550 and corresponding to the chemical formula C8H13F3N3O11P2. The isotopic abundance was also in agreement (5 ppm) with this chemical formula. The fragmentation using PRM mode (Rs = 17,500) exhibited main fragments at m/z 78.95906, 129.01068, 159.92543 and 96.96955 (Table 2). The fragment at m/z 129.01068 corresponds to the nucleobase 5-FU. All these data strongly suggested that 5-FUDP was produced by the enzymatic reaction. Thus, our method allowed the analysis of the diphosphorylated metabolites of 5-FU.

After cell exposure to 5-FU, sugar nucleotide metabolites were also identified. The metabolite 5-FUDP-Hex was detected at a retention time of 5.1 min. This compound was only detected in negative mode at m/z 583.03832 with an isotopic abundance in agreement (5 ppm) with the chemical formula C17H26F6N3O17P2. The fragmentation using PRM mode (Rs = 17,500) exhibited main fragments at m/z 78.95909, 96.96962, 211.0153, 129.01060 and 341.01965 (Table 2). The fragments at m/z 129.01060 and 341.01965 corresponded to the nucleobase 5-FU and the nucleoside 5-FUMP, respectively. The metabolite 5-FUDP-HexNaC was identified with a retention time of 5.5 min. This compound was detected in negative and positive modes at m/z 624.06487 and 626.07942, respectively. The signal intensity was about 3.5 times higher in negative mode. The exact mass and the isotopic abundance were in agreement (5 ppm) with the chemical formula C17H26F6N3O17P2. In negative ionization mode, the fragmentation using PRM mode (Rs = 17,500) exhibited main fragments at m/z 78.95911, 158.92535, 227.95718, 96.96967 and 129.01064 (Table 2). The fragment at m/z 129.01064 corresponds to the nucleobase 5-FU. These two metabolites are assigned as hexose (or hexosamine) because the mass spectrometer could not decipher if the sugar is glucose, mannose or galactose (or glucosamine, mannosamine or galactosamine) [35]. To conclude, the present LC-MS-HRMS method allows the analysis in a single run of all metabolites of 5-FU described in the anabolism phase of this drug. An extracted chromatogram of each analyte is presented in Fig. 3.

3.2. Evaluation of the method

3.2.1. Linearity

Analysis of intracellular content of HCT116 cells exposed to 5-FU revealed the presence of 5-FUr, 5-FUMP, 5-FdUMP, 5-FdUDP, 5-FUDP-Hex, 5-FUDP-HexNaC, 5-FdUr and 5-FdUMP. Cellular contents...
corresponding to \(0.5 \times 10^6\), \(1 \times 10^6\), \(2 \times 10^6\) and \(3 \times 10^6\) cells previously exposed to 50 \(\mu M\) 5-FU were prepared. As shown in Figs. 4A and B, the present method is linear since responses of each metabolite of 5-FU increased proportionally with the number of cells. The linearity of the method was also observed with the proportionality of the response as a function of the increasing quantities injected after having spiked a fixed number of cells with increasing concentrations of metabolites (Fig. S1). Moreover, RNA extracts of 0.2, 0.5, 1 and 3 \(\mu g\) were prepared and response of 5-FUrd was linear since it increased proportionally with the quantity of RNA (Fig. 4C).

3.2.2. LOD and matrix effect

To determine the analytical performances of our method and its interest for various applications around 5-FU, we determined the LOD of metabolites available as pure compounds. The LOD was equal to 12 pg injected on column for 5-FdUrd and 5-FUrd, 150 pg injected on column for 5-FUMP, 5-FdUMP, 5-FUTP and 5-FdUTP.

The matrix effect was evaluated for cellular contents, DNA and RNA extracts (Table 3). Depending on the compounds and biological matrices, the matrix effect is variable but is overall more important for nucleotides. No ion suppression due to RNA or DNA extracts constituents was observed by post-column infusion for 5-FU.
FUdR and 5-FdUrd. An ion suppression equal to 10% was observed for 5-FUMP and 5-FdUMP (Fig. S2).

3.3. Applications

3.3.1. Intracellular anabolism of 5-FU

In cellular content of HCT116 cells exposed to 10 or 50 μM 5-FU or 5-FdUrd, the 8 following metabolites of the anabolism phase were observed: 5-FUr, 5-FUMP, 5-FUDP, 5-FUTP, 5-FdUr, 5-FdUMP, 5-FdUDP-Hex and 5-FUDP-HexNAc. The two metabolites 5-FdUDP and 5-FdUTP were not detected in cellular contents, neither after 5-FU nor 5-FdUrd exposure. This absence of response was confirmed by using another cell line, HepaRG cells. In HCT116 and HepaRG cells, nucleotide metabolites of 5-FU were clearly predominant compared to nucleoside forms (nucleotides/nucleosides ratio > 10). Under the same exposure conditions, the proportion of each nucleotide metabolite of 5-FU depended on cell lines. The metabolite 5-FUTP was the most abundant in HCT116 cells, followed by 5-FUDP and then 5-FUMP while in HepaRG cells the distribution was reversed with the predominance of 5-FUMP. After cell exposure to 50 μM 5-FdUrd, all the ribonucleotide metabolites were found at the same amount than after 5-FU exposure, indicating that thymidine phosphorolase catalyzes a high proportion of 5-FdUrd to 5-FU (Fig. 1).

After cells exposure to 50 μM 5-FU for 24 h, the production of nucleotide metabolites of 5-FU was very important in HCT116 cells since the sum of the nucleotide metabolites (5-FUMP + 5-FUDP + 5-FUTP) was equivalent to that the endogenous nucleotides of uridine (UMP + UDP + UTP). In HepaRG cells the endogenous nucleotides remained in the majority (ratio 5/1).

3.3.2. Inhibition of TS by 5-FdUMP

TS is an enzyme that catalyzes the transformation of dUMP to TMP. Inhibition of TS by 5-FdUMP is one of the main mechanisms of action of 5-FU [1]. After exposure to 50 μM 5-FU, an increase of dUMP (about 5000 fold) and dUDP (about 10 fold) was shown. It was associated with a decrease of TMP (about a factor 5) and TTP (about a factor 7).

3.3.3. Analysis of 5-FU metabolites in RNA and DNA

The study of the incorporation of 5-FU into DNA and RNA can be performed by either (deoxy-)nucleoside or (deoxy-)nucleotide metabolites analysis. As described above in Section 3.1.3, it appears more relevant, according to the LOD and matrix effect, to analyze metabolites of 5-FU as nucleoside forms rather than nucleotide forms. In this case, in order to verify the completeness of the dephosphorylation step, UMP15N was added before addition of phosphatase. After incubation, it was only observed Urde15N whereas no UMP15N was detected.

RNA (3 μg) from non-treated cells or cells exposed to 10 or 50 μM 5-FU or 5-FdUrd was analyzed. Incorporation of 5-FU into RNA was detected both from dephosphorylated (5-FUr) and phosphorylated (5-FUMP) extracts. The rate of incorporation of 5-FU or 5-FUMP into RNA was similar when cells were exposed either to 5-FU or to 5-FdUrd at the same concentration. The amount of 5-FUr incorporated in RNA increased with the dose of 5-FU. This rate of incorporation was determined to 6–8 pmol/nmol of Urd and 15–17 pmol/nmol of Urd after treatment with 10 or 50 μM 5-FU, respectively. Thus, a factor of 2.5 was observed according to the cell exposure to 10 μM and 50 μM 5-FU.

DNA (3 μg) from untreated cells or cells treated with 10 or 50 μM 5-FU was also analyzed. Since a lower incorporation of 5-FU metabolite was previously described into DNA in comparison to RNA and a lower analytical response of nucleotides in comparison to nucleosides, only dephosphorylated DNA extracts were analyzed [25]. In this condition, no signal was observed for 5-FdUrd, indicating a lower incorporation of 5-FU into DNA than into RNA. Exposure of the cells to 50 μM of 5-FdUrd also failed to result in 5-FU detection from 3 μg of DNA extracts. In the present study, incorporation of 5-FU into DNA was only observed after exposure of cells to 50 μM for 24 h and when 30 μg DNA was analyzed. The rate of incorporation of 5-FU into DNA was assessed to 0.10–0.20 pmol/nmol of thymidine. Thus, the rate of incorporation of metabolites of 5-FU into RNA was 50–100 fold higher than the rate of incorporation into DNA.

3.3.4. Determination of incorporation rate of 5-FU into sub-units of RNA

It was previously demonstrated that 5-FU is incorporated into different RNA types [4]. Kanamaru et al. [36] previously showed, using L1210 cells and radiolabelled 3H-5FU, that the drug is
incorporated into 18 S and 28 S sub-units of rRNA. With our method, we confirmed the incorporation of 5-FU into 18 S and 28 S rRNAs. In humans, the 18 S and 28 S RNA molecules are composed of 1869 and 5035 nucleotides, respectively [37]. In 18 S and 28 S rRNA extracts, endogenous nucleosides (pseudoUrd, Urd, A, C, G) and 5-FUrd were quantified (between-day repeatability and accuracy results are presented in Table S1). When cells were exposed to 50 μM 5-FU for 24 h, 5-FUrd represented 0.40% and 0.33% of endogenous nucleosides corresponding to 7.5 ± 0.4 and 16.7 ± 0.2 molecules of 5-FUrd into 18 S and 28 S rRNA molecules, respectively (n = 3).

3.3.5. Inhibition of PS by 5-FU in rRNA extracts

PseudoUrd is the most abundant post-transcriptional modified nucleoside present in RNAs and plays an important biological role in different classes of RNAs [38]. The isomerization of Urd to PseudoUrd by PS leads to a C-C bond between the ribose and the Urd. The present method allows the analysis of pseudoUrd as well as pseudoUMP in dephosphorylated and phosphorylated RNA extracts, respectively. As described above for metabolites of 5-FU, the analytical response is higher for pseudoUrd than for pseudoUMP with a matrix effect clearly lower for nucleoside compared to nucleotide form. Another analytical interest in measuring pseudoUrd rather than pseudoUMP is that Urd and pseudoUrd were well separated chromatographically (6.0 min for pseudoUrd and 7.0 min for Urd). On the contrary, UMP and pseudoUMP were co-eluted (4.3 min), thus requiring the fragmentation of the two compounds to distinguish them (specific ion after positive ionization at m/z 97,02837 and m/z 125,03454 for UMP and pseudoUMP, respectively). Hengesbach et al. [39] demonstrated that 5-FU is a potent inhibitor of PS activity. As shown in a kinetic assay, the levels of pseudoUrd in RNA extracts decreased while incorporation of 5-FUrd increased when cells were exposed to 5-FU (Fig. 5).

![Fig. 4.](image)

**Fig. 4.** Linearity of the method according to the number of cells and the quantity of RNA extract. (A) Response ratio of 5-FUrd, 5-FUMP, 5-FUDP, 5-FUDP-Hex and 5-FUDP-HexNAc as function of the number of cells, (B) response ratio of 5-FdUrd and 5-FdUMP as function of the number of cells, and (C) response ratio of 5-FUrd as function of the quantity of RNA extract. IS: internal standard.

Table 3

| Matrix          | Metabolites | Matrix effect (%) |
|-----------------|-------------|-------------------|
| Cellular content| 5-FUrd      | +20               |
|                 | 5-FUMP      | -24               |
|                 | 5-FUTP      | +20               |
|                 | 5-FdUrd     | +20               |
|                 | 5-FdUMP     | +50               |
| RNA extract     | 5-FUrd      | <10               |
|                 | 5-FUMP      | -56               |
| DNA extract     | 5-FdUrd     | <10               |
|                 | 5-FdUMP     | -35               |

4. Discussion

5-FU is an “old” drug that was developed in the 1960s. However, it is still the gold standard for first-line treatment of colorectal cancer and widely used in other cancers. The medical and scientific interest remains present for this compound and many recent works focused on the study of new targets or mechanisms of chemoresistance [40–43]. Thus, it is relevant to propose a new sensitive and selective LC-MS-HRMS method, allowing the analysis of the anabolic metabolites of 5-FU and their incorporation into different cellular organelles.

4.1. Analytical method

Numerous methods reported the analysis of anabolic metabolites of 5-FU. However, the majority of them only focused on nucleoside metabolites of 5-FU [7–9,11,14,44,45]. Chromatographic analysis of nucleoside forms is easier by using, for example, reversed stationary phase with mobile phase based on methanol, acetonitrile and water [11,44]. Analysis of nucleotide forms is more challenging due to the poor retention of compounds on reversed stationary phase caused by the polarity of the phosphate moiety [33]. To overcome this issue, our separation was performed with a porous graphitic carbon (PGC) column. Four interactions could occur between the PGC stationary phase, mobile phase and compounds: hydrophobic or dispersive, electronic, steric and redox interactions [46]. This stationary phase has been already successfully used for analysis of endogenous nucleotides and nucleoside analogues [33,47]. A reverse phase column could be used, but an
ion pairing agent has to be added in the mobile phase to ensure retention of di- and tri-phosphorylated forms [12]. Anion exchange columns also allowed a good separation of metabolites of 5-FU even nucleotide forms [16,17]. Unlike Ciccolini et al. [12] and Derissen et al. [17], who used high levels of salts or ion-pairing agents, the chromatographic separation in our method was performed without these additives which are known to hamper mass spectrometric detection. Thus our method is able to separate nucleoside forms and nucleotide forms of metabolites of 5-FU without any inconvenience for mass spectrometric detection.

In our conditions, the response with ESI in negative mode was higher for the ten metabolites of 5-FU than in positive mode. This response gap is much more pronounced with nucleoside metabolites than with nucleotides. In addition, it was observed that, with the same composition of mobile phase, endogenous nucleotides have a higher response in positive mode [33]. In a mobile phase containing ammonium acetate, acetonitrile and water, Jansen et al. [47] showed a higher ionization in negative mode for 2',2'-difluorodeoxouridine (dFdU) and in positive mode for its phosphorylated metabolites (dFdUMP, dFdUDP and dFdUTP). In an acidic mobile phase, 5-fluoro-2'-deoxycytidine was ionized in positive mode [48]. We expected that our mobile phase at pH 10 and the presence of the nucleobase enhanced ESI negative ionization. Mass fragmentation after negative ionization exhibited one or more fragments containing the fluorine for all of the metabolites, thus ensuring the selectivity of the detection with endogenous nucleotides. The cleavage of the glycosidic bond leading to a fragment at m/z 129.0106 corresponding [M–H]⁻ 5-FU ion was observed for all the metabolites except 5-FdUTP. The cleavage of the ribose ring leading to a fragment at m/z 171.0211 and 155.0262 was shown for 5-FUrd and 5-FdUrd, respectively, as previously observed [13]. Mass fragmentation spectrum of 5-FdUTP exhibited a fragment at m/z 404.9905, with a relative abundance of 5%, corresponding to 5-FdUDP. Fragments at m/z 78.9591, 96.9696 and 158.9524 corresponding to the phosphate moieties were observed for nucleotide and sugar forms. Mass spectra after positive ionization are much more informative and specific to 5-FU since only fragments at m/z 97.0284 and 81.0336 corresponding to the ribose and deoxyribose moiety respectively were observed (data not shown). The study of matrix effect has shown an enhancement of ionization for some metabolites of 5-FU in intracellular content while the same metabolites encountered no matrix effect or an ion suppression in DNA or RNA matrix. Matrix effect may be due to additives present in the mobile phase, type of ionization source, but also to co-eluted compounds and depends on compounds analyzed. In nucleic acids a lower LOD was obtained for 5-FdUrd and 5-FdUdp in comparison to 5-FUMP and 5-FdUMP. Firstly, this is due to a better response with nucleosides than with nucleotides. Secondly, no inhibitory matrix effect was measured in nucleic acids extracts after dephosphorylation while a matrix effect greater than 30% was observed in cellular contents, neither after 5-FU nor 5-FdUrd exposure. Ciccolini et al. [12], with HT29 cells, have observed these two metabolites only after addition of d-Inosine in the cell medium in order to stimulate thymidine phosphorylase. In mammalian cells, dUMP is phosphorylated by thymidylate kinase and by UMP-CMP kinase whereas UMP is only phosphorylated by UMP-CMP kinase. The activation of anticancer nucleoside analogues such as 5-FU is dependent on these enzymes [34]. It was previously demonstrated that UMP is the best enzyme substrate (of the order of 50 times) than dUMP [34,49]. Thus, these data may explain the high production of 5-FUDP rather than 5-FdUDP. By analyzing the cellular content, the imbalance in deoxynucleotide pool according
5. Conclusion

We developed an LC-MS-HRMS method for the simultaneous determination of the ten metabolites (nucleoside, nucleotide and sugar nucleotide) of the anabolism phase of 5-FU. The analytical conditions were optimized to obtain an excellent sensitivity and selectivity. The presented method is suitable for determining the metabolites of 5-FU in cell extracts and to study the incorporation of 5-FU metabolite into DNA and RNA. Moreover, as the method allows in the same run the analysis of endogenous nucleosides and nucleotides, we were able to accurately determine the incorporation rate of metabolites of 5-FU into sub-units rRNA and the consequence of thymidylate synthase and pseudouridine synthase inhibition by 5-FU. In view of these results, the LC-MS-HRMS method could also be used to determine metabolites and incorporation of 5-FU into DNA and RNA in target tissues such as tumor.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2020.04.001.

References

[1] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Canc. 3 (2003) 330–338.
[2] N. Tsesmetzis, C.B.J. Paulin, S.G. Rudd, et al., Nucleobase and nucleoside analogues: resistance and Re-sensitisation at the level of pharmacokinetics, pharmacodynamics and metabolism, Cancers (Basel) 10 (2018), 240.
[3] T. Samuelsson, Interactions of transfer RNA pseudouridines syntheses with RNAs substituted with fluorouracil, Nucleic Acids Res. 19 (1991) 6139–6144.
[4] W.B. Parker, Y.C. Cheng, Metabolism and mechanism of action of 5-fluorouracil, Pharmacol. Ther. 48 (1990) 381–395.
[5] G. Weckbecker, D.O. Keppler, Substrate properties of 5-fluorouridine diphospho sugars detected in hepatoma cells, Biochem. Pharmacol. 33 (1984) 2291–2298.
[6] A. Prochazkova, S. Liu, H. Friess, et al., 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.
[7] H. Ishii, M. Shimada, H. Yamaguchi, et al., A simultaneous determination method for 5-fluorouracil and its metabolites in human plasma with linear range adjusted by in-source collision-induced dissociation using hydrophilic interaction liquid chromatography-electrospray ionization-tandem mass spectrometry, Biomed. Chromatogr. 30 (2016) 1882–1886.
[8] M.J. Deenen, H. Rosing, M.J. Hillebrand, et al., Quantitative determination of capecitabine and its metabolites in colon cancer patients, Pharmacol. Res. 50 (2004) 173–179.
[9] P. Deng, C. Ji, X. Dai, et al., Simultaneous determination of capecitabine and its three nucleoside metabolites in human plasma by high performance liquid chromatography-tandem mass spectrometry, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 989 (2015) 31–40.
[10] J.L. Holleran, J.L. Eisenman, R.A. Parise, et al., LC-MS/MS assay for the quantitation of FdCyd and its metabolites FdUr and FU in human plasma, J. Pharmaceut. Biomed. 129 (2016) 359–366.
[11] J. Cicalcin, L. Peillard, C. Aubert, et al., Monitoring of the intracellular activation of 5-fluorouracil to deoxyribonucleotides in HT29 human colon cell line: application to modulation of metabolism and cytotoxicity study, Fund. Clin. Pharmacol. 14 (2000) 147–154.
[12] D. Carli, M. Honorat, S. Cohen, et al., Simultaneous quantification of 5-FU, 5-FUr, 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.
