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Simultaneous quantification of 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP and TMP in cultured cell models by LC-MS/MS

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ABSTRACT

To specifically quantify several metabolites of 5-fluorouracil (5-FU) and two endogenous monophosphate nucleotides, we developed an original method based on a liquid chromatography–tandem mass spectrometry (LC-MS/MS). This assay allowed the determination of: (i) the intracellular production of 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP) from 5-FU or 5-fluoro-2′-deoxyuridine (5-FdUrd), (ii) the impact of 5-FdUMP concentration on the intracellular 2′-deoxyuridine-5′-monophosphate (dUMP)/thymidine-5′-monophosphate (TMP) ratio, and (iii) the secretion extent of 5-FdUMP and 5-FU from human cultured cells by ABC transporters. Under our experimental conditions, cells were incubated with 5-FU or 5-FUrd. Then, cellular proteins were precipitated by methanol. This procedure provided high extraction recovery. In addition, to measure 5-FU and 5-FdUMP secretion from cells, we carried out quantification of these molecules in culture medium. Media were either directly injected (5-FU) or underwent a solid phase extraction using Oasis Wax extraction cartridge (5-FdUMP). Separation of analytes was performed on a dC18 Atlantis 3.5 μm, (100 mm × 2.1 mm i.d) column with isocratic mode using ammonium formate buffer/methanol/water (5/5/90, v/v) as mobile phase. The run time did not exceed 6.2 min. The analytes were ionized in an electrospray interface under negative ion mode. We validated the method over a range of 2.5–150 ng mL^{-1} according to the compounds. Intra- and inter-assay variability was lower than 10% over seven days. All compounds were stable in cells or in culture medium when samples were stored at −20 °C for at least two weeks, and after three freeze-thaw cycles. No matrix effect was observed in both media.

1. Introduction

The 5-Fluorouracil (5-FU) anticancer drug has been widely used for fifty years in the treatment of several human solid tumors including colorectal and breast cancers [1,2]. The pyrimidine 5-FU undergoes a complex metabolic pathway leading to the production of cytotoxic metabolites such as fluorouridine triphosphate and deoxyfluorouridine triphosphate that are respectively incorporated into RNA and DNA (Fig. 1) [3]. Among the metabolites, 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP) plays a key role. Indeed, 5-FdUMP inhibits thymidylate synthase (TS), the enzyme that transforms 2′-deoxyuridine-5′-monophosphate (dUMP) to thymidine-5′-monophosphate (TMP) that leads to intracellular depletion of thymidine [4]. Conversely, 5-FdUMP is a substrate of the efflux pumps such as ABCC5 and ABCC11 that have recently been associated with 5-FU resistance mechanisms [5,6].

Several assays described the quantification of 5-FU and its metabolites 5-fluorouridine (5-FUrd) and 5-fluoro-2′-deoxyuridine (5-FdUrd) [7–12]. However, few of them included the quantification of the pharmacologically active 5-FdUMP metabolite. Among these methods, the first one used tritium radiolabeled 5-FU ([^{3}H-5FU]) and a chromatographic separation with counter ion. The quantification of 5-FU and its phosphate metabolites was successfully developed in cell lines [13,14]. The second method for the quantification of 5-FdUMP was performed in L1210 cells using

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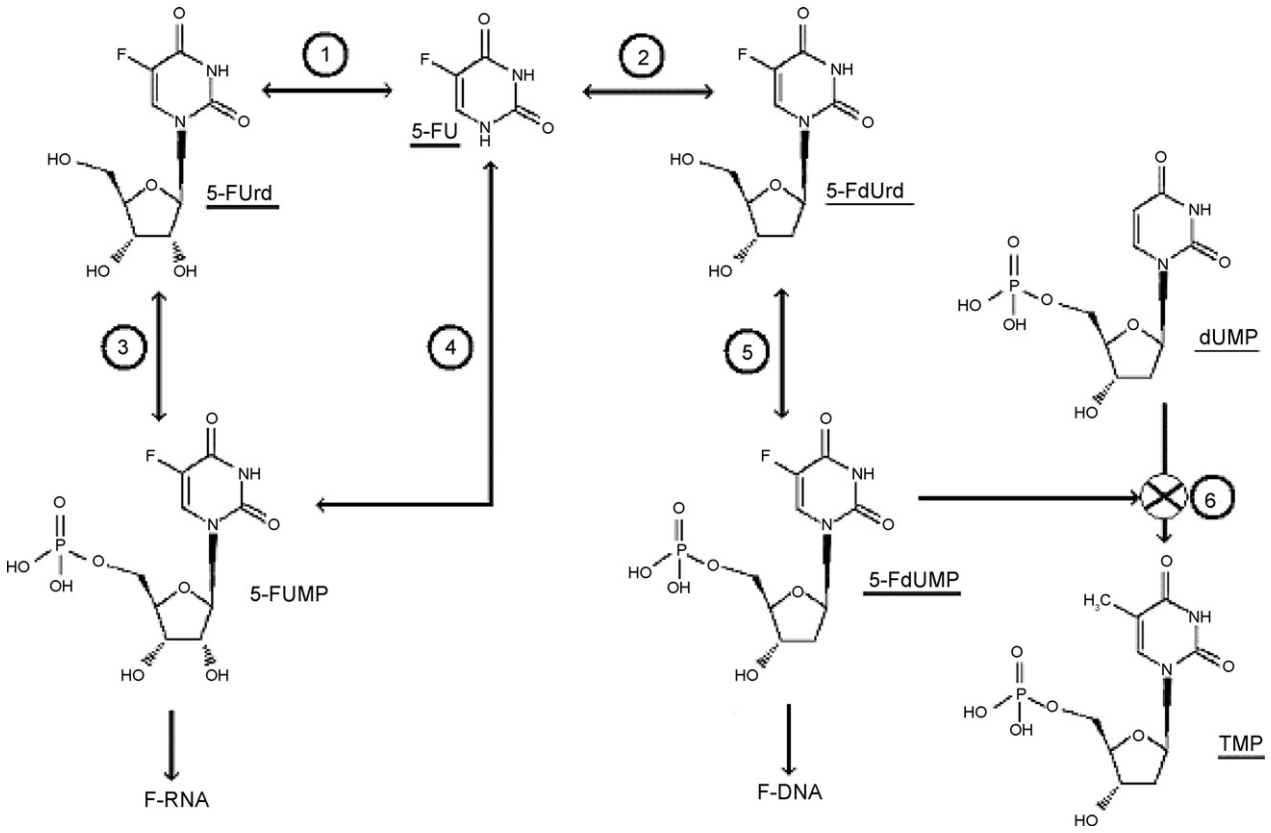

Fig. 1. Partial intracellular anabolism pathways of 5-fluorouracil (5-FU). The compounds underlined were quantified in the present study. 5-fluorouracil (5-FU), 5-fluorouridine (5-FUrd), 5-fluoro-2′-deoxyuridine (5-FdUrd), 5-fluorouridine-5′-monophosphate (5-FUMP), 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP), 2′-deoxyuridine-5′-monophosphate (dUMP), thymidine-5′-monophosphate (TMP). 1: uridine phosphorylase, 2: thymidine phosphorylase, 3: uridine kinase, 4: thymidine kinase, 5: pyrimidine phosphoribosyl transferase, 6: thymidylate synthase.

also [^{3}H-5FU], but it was time-consuming due to a pre-treatment with periodate and methylamine to eliminate ribonucleotides [15]. Other assays using LC-UV with counter ion or based on strong cation exchangers have been published for the separation of 5-FU, 5-FUrd, 5-FdUrd and 5-FdUMP in plasma. However, poor sensitivity was obtained and interfering peaks from endogenous compounds were observed, especially for 5-FdUMP [16,17]. The latest method was based on capillary electrophoresis coupled with UV detection but it could not detect 5-FdUMP in cells due to poor sensitivity [18].

In the present study we have developed an assay in cultured cell models to simultaneously quantify 5-FU derivatives (5-FUrd, 5-FdUrd, 5-FdUMP) and endogenous molecules (dUMP and TMP). We assessed that the dUMP and TMP concentrations were modified via TS inhibition by 5-FdUMP. 5-FU and 5-FdUMP molecules were also quantified in extracellular compartment (culture medium). Here we describe a rapid, sensitive and specific method based on LC-MS/MS technology to quantify various 5-FU derivatives present in cells and effluxed culture media.

2. Experimental

2.1. Reagents and chemicals

5-Fluorouracil (5-FU), 5-Chlorouracil (5-CU), 5-fluorouridine (5-FUrd), 5-fluoro-2′-deoxyuridine (5-FdUrd), 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP), 2′-deoxyuridine-5′-monophosphate (dUMP), thymidine-5′-monophosphate (TMP), as pure standards, were purchased from Sigma (Saint-Quentin Fallavier, France). All products were stored at +4 °C in the dark, except for 5-FdUMP that was stored at −20 °C. HPLC-grade acetonitrile was obtained from Merck (Darmstadt, Germany). Acetic acid (Ultra, >99.5% pure) was supplied by Fluka (Steinhein, Germany). Methanol was from Carlo Erba (Milano, Italy). Milli-Q deionized water was used throughout the study.

2.2. Standard solutions, calibration standards and quality controls

Stock solutions (1 mg mL^{-1}) of 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP, TMP and 5-CU were prepared in methanol and stored at −20 °C. Standard solutions were made daily by further dilution of stock solutions with methanol. For calibration curves, methanol (total volume 500 μL) was spiked with 25 μL of the appropriately diluted standard solutions. Final concentrations were 2.5, 5, 7.5, 25, 50 ng mL^{-1} for TMP (7.8–388 nM), 7.5, 15, 22.5, 75, 150 ng mL^{-1} for 5-FU (57.7–1154 nM) and 5, 10, 15, 50, 100 ng mL^{-1} for 5-FUrd (19.1–382 nM), 5-FdUrd (20.3–407 nM), 5-FdUMP (15.3–307 nM), dUMP (16.2–325 nM). Quality controls (C1 and C2, respectively) were prepared at the following concentrations: 11.25 and 112.5 ng mL^{-1} for 5-FU (86.5 and 865 nM), 7.5 and 75 ng mL^{-1} for 5-FUrd (28.6 and 286 nM), 5-FdUrd (30.5 and 305 nM), 5-FdUMP (23 and 230 nM), dUMP (24.4 and 244 nM), 3.75 and 37.5 ng mL^{-1} for TMP (11.7 and 117 nM). Blank cell samples without 5-FU derivatives were also carried out (dUMP and TMP are endogenous compounds). All samples were subjected to the sample procedure as described below.

2.3. Sample preparation

2.3.1. Cellular quantification of 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP and TMP.

Samples diluted in 500 μL of methanol were used for calibration curves, quality control or cell incubation. 5-CU was used as

internal standard and added at 200 ng mL^{-1}. Then samples were centrifuged at 12,000 × *g* for 5 min at +10 °C. The supernatant was removed and evaporated to dryness under a stream of nitrogen. The residues were resuspended in 300 μL of water and 10 μL was injected in the HPLC device.

2.3.2. Quantification of 5-FU in culture medium.

Quantification of 5-FU was performed by directly injecting 10 μL of the cell culture medium into the LC-MS/MS device. 5-CU was used as internal standard and added at 500 ng mL^{-1}.

2.3.3. Quantification of 5-FdUMP in cell culture medium.

In this case, dUMP was used as internal standard. We carried out a solid phase extraction (SPE) according to the following procedure: an Oasis Wax (60 mg), extraction cartridges (Waters, Milford, USA) was conditioned under vacuum with 2 mL of methanol and 2 mL of acetic acid (1%). dUMP (500 ng mL^{-1}) was added to 300 μL Dulbecco's Modified Eagle's Medium (DMEM), then the mixture was applied to an adsorbent SPE column. After a slow percolation, the cartridge was successively washed with 1 mL of acetic acid (1%), 2 mL of water and 1 mL of methanol/ammonium hydroxide 0.1% (30/70, v/v). The elution was performed with 1 mL of methanol/ammonium hydroxide 3% (90/10, v/v). This eluted solution was evaporated at +30 °C under a gentle stream of nitrogen. The residue was reconstituted in 300 μL of water. After vortexing, the sample was centrifuged 5 min at 4000 × *g*, and the clear supernatant was transferred to a glass vial kept at +10 °C into the autosampler and 10 μL was injected.

2.4. HPLC conditions

The high-performance liquid chromatographic system consisted of a ThermoElectron Surveyor MS pump equipped with and Surveyor autosampler injector (ThermoElectron, San Jose, USA). Samples were separated on a dC18 column Atlantis, 3.5 μm, (100 mm × 2.1 mm i.d) (Waters, Milford, USA). The separation was performed with isocratic mode using a mixture of ammonium formate buffer (5 mM, pH 4)/methanol/water (5/5/90, v/v). The mobile phase was delivered through the column (temperature maintained at +30 °C) at a flow rate of 200 μL min^{-1}.

2.5. Mass spectrometry conditions

LC-MS/MS analyses were acquired using a Quantum-Ultra (ThermoElectron, San Jose, USA) triple quadrupole mass spectrometer equipped with an Ion Max API source. The instrument was operated in negative ion mode with electrospray (ESI) source. The position (*x*, *y*, *z*) of the ESI probe was optimized with 5-FdUMP. Argon was used as collision gas at 1.5 mTorr. Spray voltage and capillary temperature were set respectively at 3.5 kV and 350 °C. Pressures for the nitrogen sheath gas, auxiliary gas and sweep gas were respectively maintained at 40, 15 and 5 units (units refer to an arbitrary value set by the X-calibur software). The $[M-H]^-$ ions of different compounds were passed through the first quadrupole (Q1), then after fragmentation, daughter ions were passed through the third quadrupole (Q3) with full-width at half maximum height of 0.7 *m*/*z* for both quadrupoles. Compounds were quantified in selected reaction monitoring (SRM) mode with 100 ms dwell time per channel. The transitions and collision energies are summarized in Table 1.

2.6. Calibration curves and validation procedure

Calibration curves were constructed by plotting the ion abundance peak area ratio (analyte/internal standard) as a function of cell culture analyte concentrations. The significance of the slope and the validity of the linear calibration curves were tested using Fisher–Snedecor's *F*-test ($p < 0.05$). Homocedasticity was statistically determined using Cochran's test ($p < 0.05$).

Table 1
Transitions monitored and collision energies used.

	Transitions (*m*/*z*)	Collision energy (eV)
5-FU	129 → 42	25
5-FUrd	261 → 129	20
5-FdUrd	245 → 155	15
5-FdUMP	325 → 195	15
TMP	321 → 195	20
dUMP	307 → 195	18
5-CU (I.S.)	145 → 42	28

For intracellular quantification, seventeen independent calibration curves were prepared. Seven runs included a calibration curve and quality control samples (QC) at two different concentrations (see above) in six replicates. For the quantification in DMEM, five runs including a calibration curve and quality control samples were performed. For each analyte, the lower limit of quantification (LLOQ) was chosen as the concentration of the lowest calibration standard. The upper limit of quantification was chosen as the concentration of the upper calibration standard.

The accuracy and precision of the assay were respectively assessed by the mean relative percentage deviation from the nominal concentrations and the within-run precision (WRP) and between-run precision (BRP). The within-run precision was determined as $WRP = 100 \times (\sqrt{MS_{wit}}/GM)$. The between-run precision was estimated as $BRP = 100 \times (\sqrt{(MS_{bet} - MS_{wit})/n}/GM)$. MS_{wit}, MS_{bet}, *n*, GM, represented the within-groups mean square, the between-groups mean square, the number of replicate observations within each run and the grand mean, respectively. These parameters were calculated using the software Statview for windows version 5.0 (SAS institute, Cary, USA).

The extraction efficiency of analytes was calculated from the QC samples. The determination was made by comparing the mean peak areas from samples obtained through the extraction procedure with those obtained from direct injection of the same amount dissolved in the mobile phase.

2.7. Stability

The stability study was carried out in duplicate and was performed from cellular samples for all the compounds using QC1 and QC2 and from DMEM for 5-FU and 5-FdUMP using two concentrations (50 and 400 ng mL^{-1}). The freeze-thaw stability was tested following three cycles at −20 °C. The auto-sampler stability at +10 °C was tested by analyzing the compounds every 2 h during 8 h. The long-term freezing stability at −20 °C was tested by re-analyzing the samples two weeks after the first analysis.

2.8. Matrix effect

To investigate the effects of the matrix components on the suppression of the ESI signal, a post-column infusion system with syringe pump (flow rate 5 μL min^{-1}) was used to deliver the different compounds (1 μg mL^{-1}). Effluent from the HPLC column combined with the infused analytes entered into the detector. Extract samples from cells or DMEM and from no extract DMEM were successively injected.

2.9. In vitro cell models

Human breast MCF7 and human kidney HEK 293T cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) under 5% CO_2 at +37 °C. All experiments were performed in

exponentially growing cells. At approximately 80% confluence, the medium was removed and replaced with FBS-free DMEM. Following incubation period with 5-FU derivates, DMEM was removed and stored at −20 °C for the quantification of 5-FU and 5-FdUMP. Cells were washed three times with ice-cold PBS and then 500 μL of methanol supplemented with internal standard was added. The MCF7 cell line was used for the development of the method in the presence of 100 μM 5-FdUrd. Experiments were performed using MCF7 or HEK 293T cell lines, respectively incubated with 5-FdUrd or 5-FU at various concentrations (0, 5, 10, 20, 50, 100 μM) and various incubation times (0, 5, 10, 15, 30, 45 min). Total protein content was determined using the Bradford Protein Assay method (Bio-Rad laboratories, CA, USA).

3. Results and discussion

3.1. Liquid chromatography

5-FU, 5-FUrd, and 5-FdUrd are hydrophilic compounds. For 5-FdUMP, dUMP and TMP, the phosphate group increases the polarity compared to their nucleoside analogues. This makes the chromatographic retention of these molecules a challenge by LC-MS since a limited number of mobile phases are suitable for both LC separation and MS detection. One way to overcome this problem is the use of a volatile ion-pair reagent as previously described for monophosphate nucleotides [13,16]. Another possibility is to perform the chromatographic separation based on hydrophilic interaction chromatography (Hilic). This method presents the advantage of requiring a high percentage of organic solvent favourable for MS detection. However, the concentration of buffer which must be present to obtain reproducible retention times and good peak shapes leads to a strong decrease in the MS response for 5-FU derivatives and endogenous molecules. This observation comes from our own experience but had also previously been reported by Siethoff et al. for the quantification of 5-FU by LC-MS/MS [19]. Finally, we chose a highly aqueous mobile phase with a dC18 Atlantis column specifically developed for this analytical condition. The buffer concentration was a critical parameter for the sensitivity of our present method (Fig. 2). Indeed, the response for all analytes decreased inversely with the buffer concentration. However, a final concentration of buffer in mobile phase at 0.25 mM allowed obtaining a good peak shape and an acceptable sensitivity. The best compromise between runtime, sensitivity and peak shape was observed with the present conditions rather than those tested

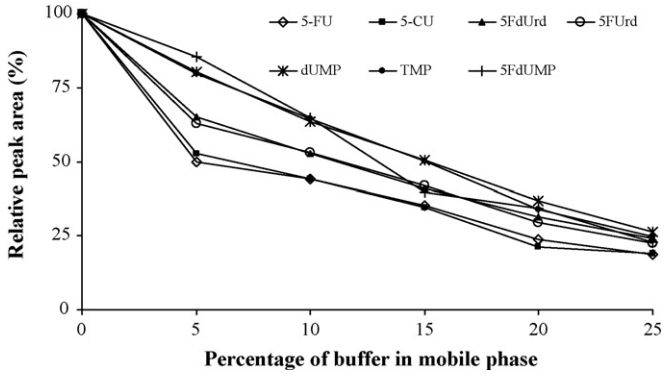

Fig. 2. Response of the mass spectrometry detector according to the composition of the mobile phase.
The first condition was methanol/water (5/95, v/v). Then for other conditions, methanol was maintained at 5% and ammonium formate buffer (5 mM, pH 4) was introduced from 5 to 25% (final concentration through the analytical column from 0.25 to 1.25 mM). The data represents the peak area ratio between experimental conditions and the basal condition (without buffer).

with Hilic column. The chromatography was performed with isocratic condition and the run time did not exceed 6.2 min. The mobile phase was initially discarded, and then automatically switched to the detector at 2.3 min and the compounds were detected from 3 to 5.2 min. Following this procedure, using LC-MS/MS analysis, no interfering endogenous peaks were observed from cells (Fig. 3).

Direct injection of DMEM into the LC-MS/MS device allowed monitoring 5-FU, 5-FdUrd, 5-FUrd and 5-CU. However, under this condition, monophosphate compounds show very broad peaks. DMEM includes numerous constituents (amino acids, minerals, vitamins, . . .) and some of them are at a high concentration such as glucose, phosphate buffer, NaCl. We suggested that DMEM contents altered the chromatographic behaviour and the mass response (see matrix effect) of highly hydrophilic monophosphate derivatives while other non-monophosphate compounds were not affected. Consequently, we performed a SPE prior to the chromatographic analysis for the quantification of 5-FdUMP in DMEM.

3.2. Mass spectrometry

The optimal mass spectrometry conditions for the detection of all compounds were achieved in negative ion mode. All compounds predominantly formed deprotonated ions ($[M-H]^-$). The fragmentation of 5-FU ($[M-H]^-$ ion at m/z 129) consists in a cleavage of the ring leading to a daughter ion at m/z 42 as previously described [20]. For the monophosphate compounds the major ion produced by fragmentation was the deoxyribose monophosphate ion (m/z 195) obtained by cleavage of the glycosidic bond. The SRM transitions were 307 → 195, 321 → 195 and 325 → 195 for dUMP, TMP and 5-FdUMP, respectively (data not shown). The fragmentation also yields the expected [M-195] fragment ion corresponding to the ion of the uracile base for dUMP (m/z 111), the thymidine base for TMP (m/z 125) and to 5-FU for 5-FdUMP (m/z 129). This fragmentation pathway was the same as the one described for stavudine-monophosphate [21]. The fragmentation of 5-FUrd and 5-FdUrd corresponded to the cleavage of the glycosidic bond yielding an ion at m/z 129 or the cleavage of the sugar ring leading to respectively an ion at m/z 155 and 171 for 5-FdUrd and 5-FUrd. Although the fragmentation pathway was the same for both compounds, the intensities of the daughter ions reported in Fig. 4 were very different.

3.3. Method validation

For analysis of endogenous compounds, the choice of the sample matrix for the preparation of calibration standards and QC samples is challenging. During the method development numerous, cell samples were spiked at various concentrations with exogenous compounds 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP and 5-CU (I.S.). Then the results were compared with those obtained when methanol was spiked at the same concentrations. In both cases, the sample preparation was carried out under the same procedure. No significant difference was observed between the two matrices. Consequently, calibration curves and QC samples for all the analytes, including endogenous compounds dUMP and TMP, were performed from the methanol matrix.

The calibration curves were constructed by analyzing data as plots of peak area ratio of analyte/internal standard versus the analyte concentration. Results showed that a weighting factor had to be applied. The weighting factor 1/[concentration] was found to provide the best fit. Then, raw data points were fitted to a linear or quadratic least-squares regression curve. The determination coefficient (r^2) of the linear regression was at least equal to 0.992 for all compounds. Calibration curves for analytes on seventeen individual days are presented in Table 2. For all concentrations the inter-day precision (CV) was lower than 10% and the accuracy was in the

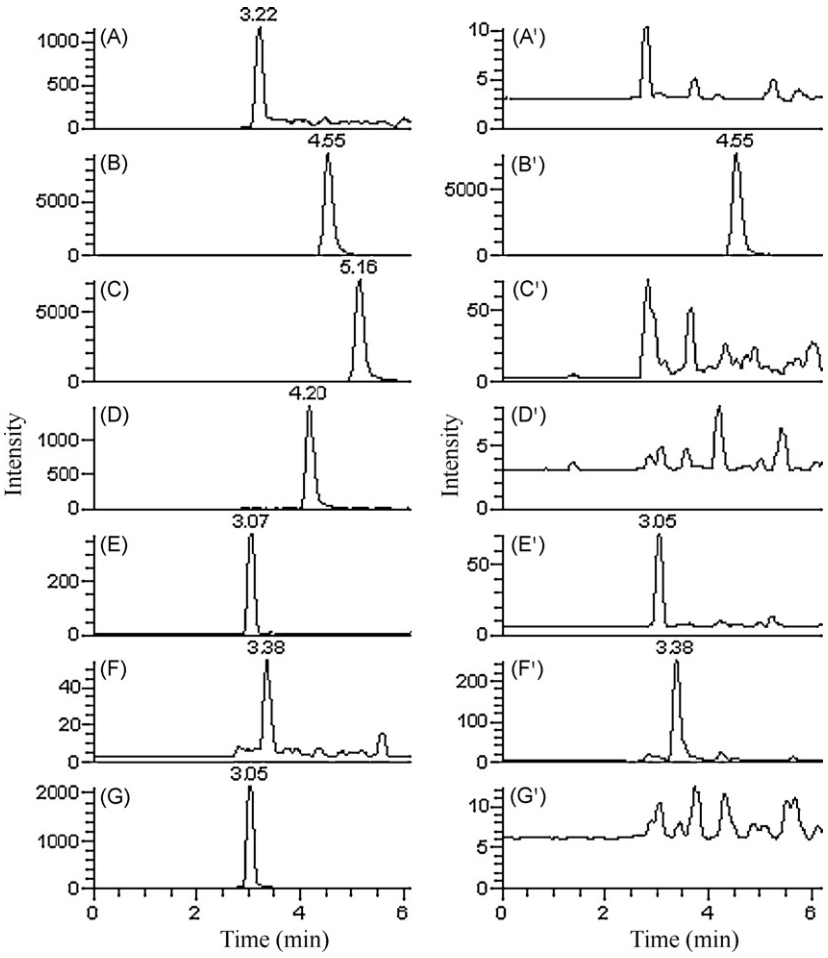

Fig. 3. Chromatograms obtained after incubation for 15 min of MCF7 cells with (left) 5-FdUrd and without (blank) (right) 5-FdUrd.
(A and A′) 5-fluorouracil (5-FU); (B and B′) 5-Chlorouracil (5-CU) (I.S.); (C and C′) 5-fluoro-2′-deoxyuridine (5-FdUrd); (D and D′) 5-fluorouridine(5-FUrd); (E and E′) 2′-deoxyuridine-5′-monophosphate (dUMP); (F and F′) thymidine-5′-monophosphate (TMP); (G and G′) 5-fluoro-2′-deoxyuridine-5′-monophosphate (5-FdUMP).

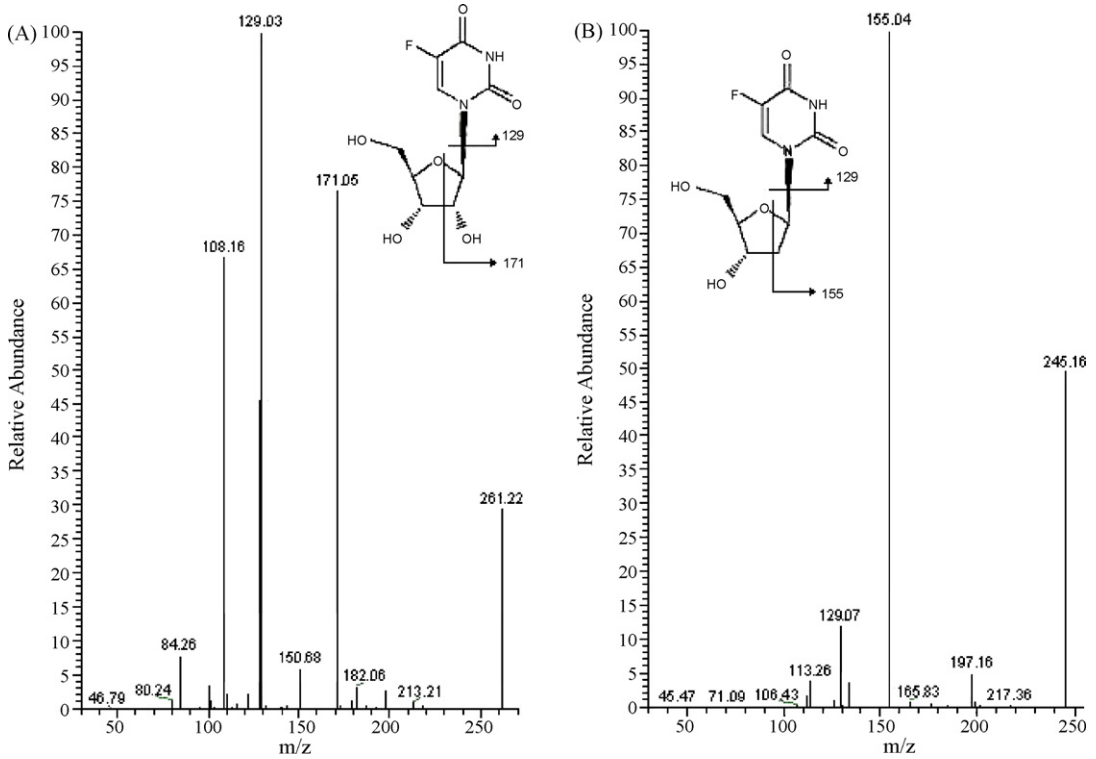

Fig. 4. Product ion mass spectrum (partial fragmentation) of 5-fluorouridine (5-FUrd–[M−H]$^-$: 261.22) (A) and 5-fluoro-2′-deoxyuridine (5-FdUrd–[M−H]$^-$: 245.16) (B).

Table 2
Inter-day validation of the intracellular determination of 5-FU, several metabolites of 5-FU and dUMP and TMP. Data from seventeen calibration curves prepared as a single replicate and analyzed on seventeen different days.

Concentration (ng mL^{-1})		Precision (%) (between-run)	Accuracy (%)
Spiked	Found (mean ± SD, 17 days)		
5-FU			
7.5	7.2 ± 0.7	9.2	96.1
15	15.9 ± 1.2	7.7	105.8
22.5	22.8 ± 1.6	6.9	101.2
75	71.8 ± 4.5	6.3	95.7
150	152.5 ± 4.4	2.9	101.7
5-FUrd			
5	4.9 ± 0.4	8.6	97.4
10	10.3 ± 0.8	7.6	103.3
15	15.3 ± 1.3	8.2	102.0
50	49.7 ± 1.7	3.4	99.5
100	100.0 ± 0.6	0.6	100.0
5-FdUrd			
5	5.1 ± 0.4	7.1	101.2
10	10.1 ± 0.6	6.0	100.8
15	14.9 ± 1.3	8.5	99.7
50	48.2 ± 2.3	4.9	96.5
100	101.9 ± 2.4	2.4	101.9
5-FdUMP			
5	5.8 ± 0.4	6.0	116.4
10	9.7 ± 0.7	7.4	96.8
15	14.0 ± 1.1	7.5	93.4
50	47.4 ± 3.7	7.7	94.7
100	103.8 ± 3.9	3.8	103.8
TMP			
2.5	2.5 ± 0.2	9.4	98.1
5	5.2 ± 0.5	8.7	104.3
7.5	7.5 ± 0.6	8.0	99.6
25	25.0 ± 0.6	2.5	100.1
50	50.0 ± 0.2	0.4	100.0
dUMP			
5	5.0 ± 0.4	7.2	99.4
10	10.5 ± 0.7	6.6	105.2
15	14.3 ± 1.3	9.2	95.5
50	50.5 ± 1.1	2.3	100.9
100	99.8 ± 0.4	0.4	99.8

Table 3
Assessment of accuracy and precision. Data from six replicates for each concentration and analyzed on seven different days.

Concentration (ng mL^{-1})		Precision (%)		Accuracy (%)
Spiked	Found (mean ± SD)	Within-run	Between-run	
5-FU				
11.25 (C1)	11.5 ± 1.1	8.4	5.0	101.9
112.5 (C2)	114.2 ± 6.8	4.9	3.5	101.5
5-FUrd				
7.5 (C1)	7.5 ± 0.7	7.8	6.1	100.7
75 (C2)	73.5 ± 4.2	5.3	2.1	98.0
5-FdUrd				
7.5 (C1)	7.2 ± 0.6	5.8	5.7	96.9
75 (C2)	73.5 ± 3.9	4.2	3.5	98.0
5-FdUMP				
7.5 (C1)	7.5 ± 0.7	7.0	5.4	99.6
75 (C2)	70.0 ± 6.0	8.0	3.7	93.4
TMP				
3.75 (C1)	3.8 ± 0.3	7.0	2.9	101.5
37.5 (C2)	36.5 ± 1.7	4.5	1.4	93.3
dUMP				
7.5 (C1)	7.2 ± 0.7	8.1	5.8	95.6
75 (C2)	74.9 ± 5.5	6.5	3.4	100.0

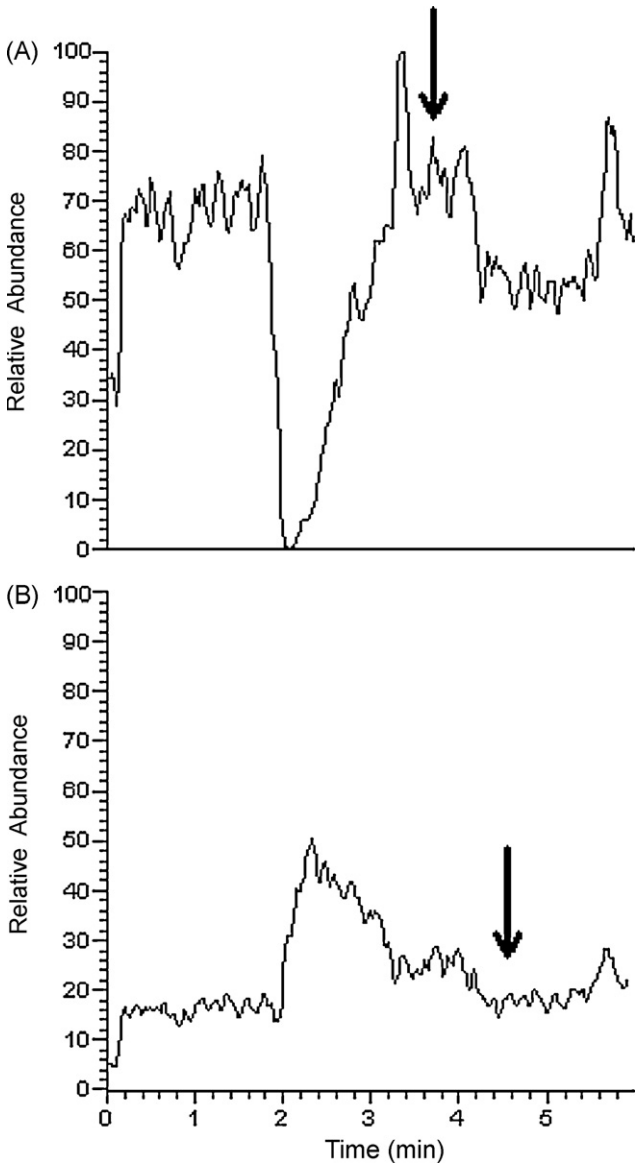

Fig. 5. Ionization suppression study of 5-FU (A) and 5-CU (B) from no extract DMEM sample. Arrow indicates the retention time of compounds.

range of 93.4–105.8% except for 5-FdUMP at 5 ng mL^{-1}. The resulting assay precision and accuracy data are presented in Table 3. The within-run precision of the assay was less than 8.5% for each concentration on two QC samples. The between-run precision of the assay was less than 6.5% for all the QC samples. Assay accuracy was in the range of 93.3–101.9%. For all compounds, the lower limit of quantification was set at the lowest calibration standard. At this concentration, precision was within 20% and accuracy between 80 and 120%.

The extraction recovery was studied from cellular matrix. 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP concentrations were measured in cells or in mobile phase spiked at the concentration of QC1 and QC2 ($n = 6$). For both concentrations tested and for all compounds, the recovery was greater than or equal to 90% except 5-FU and 5-FdUrd at QC2. This latter compound gave a slightly lower recovery rate at 85%.

The quantification of 5-FU and 5-FdUMP was studied between 25 and 500 ng mL^{-1} in DMEM. For both compounds the within- and the between-run precisions were less than 6.0% and accuracy was less than 5.0%.

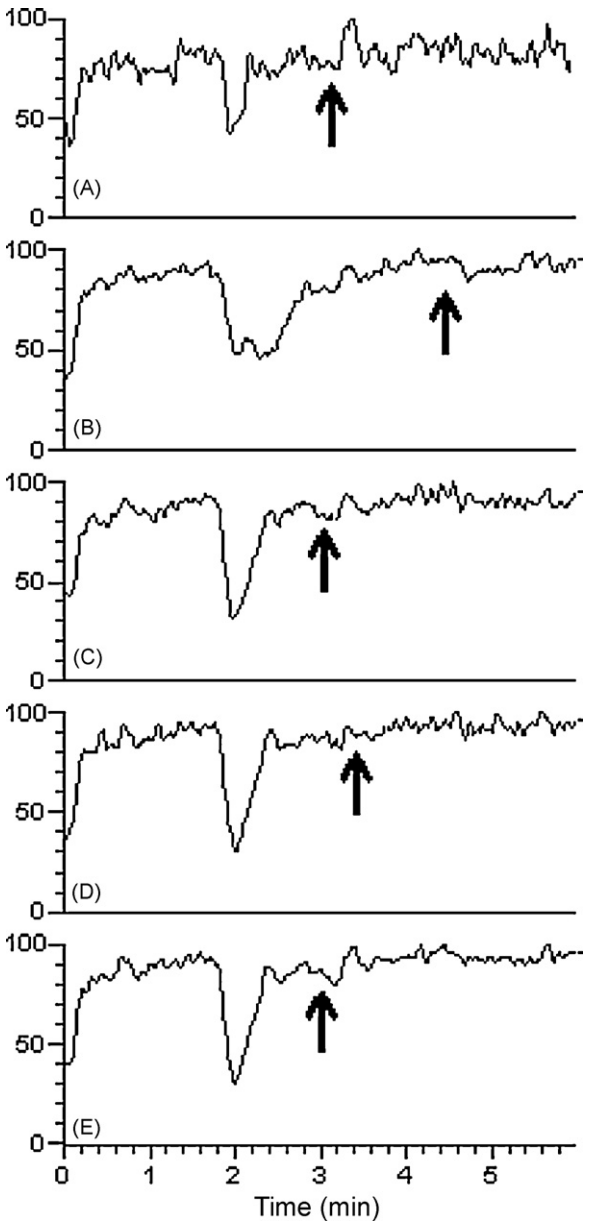

Fig. 6. Ionization suppression study of 5-FU (A), 5-FdUrd (B), dUMP (C), TMP (D) and 5-FdUMP (E) from cellular sample. Arrow indicates the retention time of compounds.

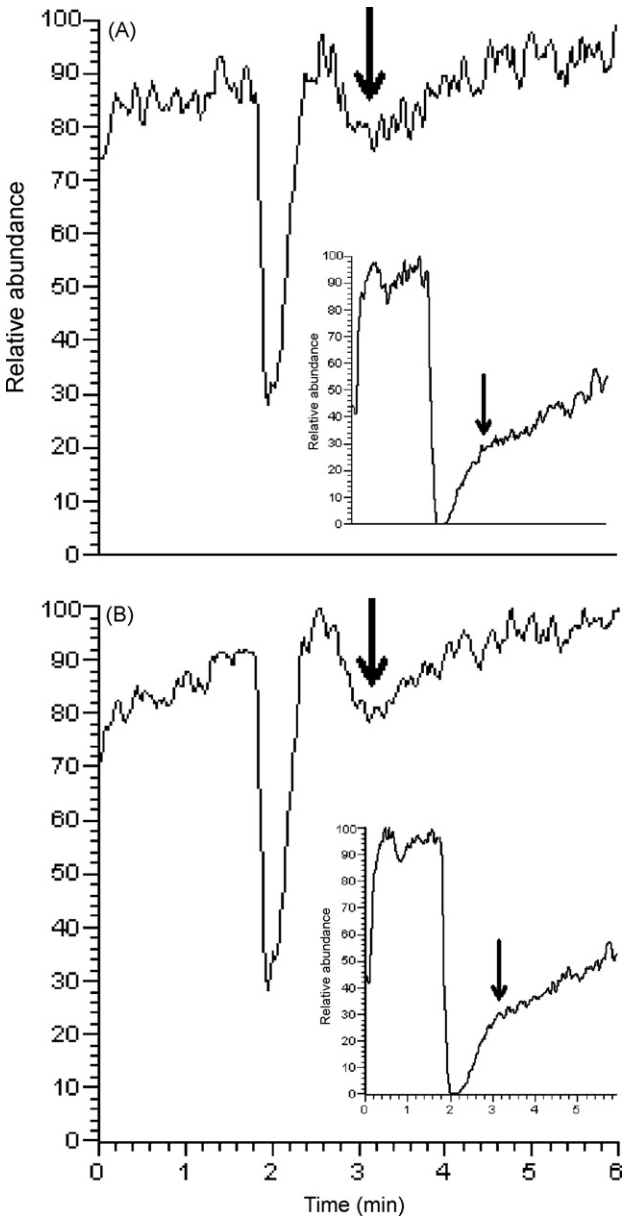

Fig. 7. Ionization suppression study of dUMP (A), and 5-FdUMP (B) from extract DMEM sample. Inset shows the ionization suppression from no extract DMEM sample.
Arrow indicates the retention time of compounds.

3.4. Assessment of stability

No significant degradation (<10%) was observed after three freeze-thaw cycles for concentrations tested from cellular samples as well as from cell culture medium (5-FU and 5-FdUMP). After extraction, when glass vials were maintained in the autosampler at +10 °C, all compounds from both matrices did not show degradation for at least 8 h. This allowed analyzing over 65 samples within a single run. Finally, no significant degradation (<10%) was observed when cellular or DMEM samples were kept at −20 °C for at least two weeks.

3.5. Matrix effect

No matrix effect was observed from no extract DMEM sample for 5-FU and 5-CU analysis (Fig. 5) or from cellular sample (Fig. 6). A broad region of ionization suppression was observed for monophosphate compounds when DMEM sample was analyzed without extraction, while SPE procedure allowed clearing this phenomenon (Fig. 7).

3.6. Application

As shown in Fig. 8, the present methodology allows following the evolution of intracellular concentration of the compounds of interest (see Fig. 1 for the metabolic pathway). The amount of 5-FdUMP increased with time linearly during 30 min (Fig. 5A) and with the concentration of substrate 5-FdUrd (Fig. 5B). Furthermore, the yield of 5-FdUrd and 5-FUrd increased with time when 5-FU is used as substrate (Fig. 5C). Interestingly, the inhibition of TS by 5-FdUMP was observed by monitoring an accumulation of dUMP and a slight decrease in TMP (Fig. 5D). This assay was also used to study the efflux transport of 5-FU and 5-FdUMP by quantification of both compounds in the cells and in DMEM incubation medium (data not shown) [22].

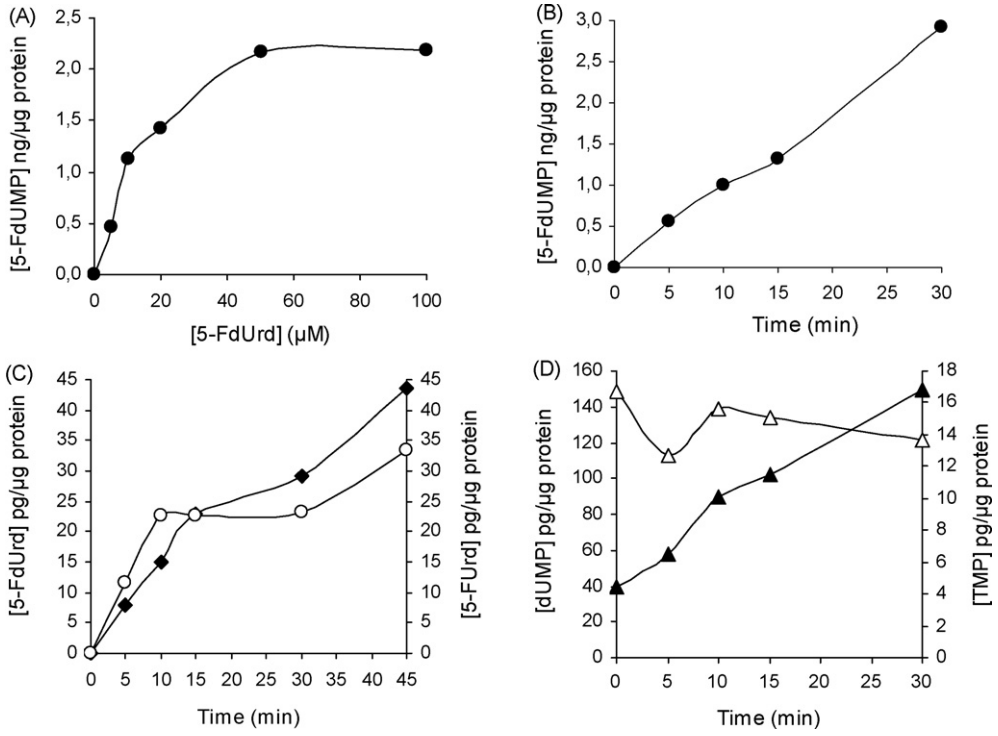

Fig. 8. Evolution of intracellular concentration of several compounds according to time and substrate concentration in MCF7 and HEK 293T cells.
(A and B) Intracellular concentration of 5-FdUMP (closed circle) in MCF7 cells according to variable 5-FdUrd concentrations at a fixed 15 min incubation time (A), according to time with a fixed 5-FdUrd concentration to 20 µM (B). (C) Intracellular concentration of 5-FdUrd (open circle) and 5-FUrd (closed square) in HEK 293T cells depending on time with 20 µM 5-FU. (D) Intracellular concentration of dUMP (closed triangle–left scale) and TMP (open triangle–right scale) in MCF7 cells depending on time with 20 µM 5-FdUrd.

4. Conclusion

A LC-MS/MS methodology was developed for the quantitative determination of 5-FU, 5-FUrd, 5-FdUrd, 5-FdUMP, dUMP, TMP in cell culture systems. This method provides accuracy, specificity, sensitivity for the analytes studied, without the safety requirements associated with radioactive substance handling. The technique described includes simple sample preparation steps and better selectivity in detection compared to conventional LC–UV assays. The current methodology also has the potential to be used for the determination of compounds in other fluid such as cell culture medium. In this case, an additional step with a solid phase extraction is needed for the determination of monophosphate compounds.

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