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The achievement of mass balance by simultaneous quantification of floxuridine prodrug, floxuridine, 5-fluorouracil, 5-dihydrouracil, α -fluoro- β -ureidopropionate, α -fluoro- β -alanine using LC-MS

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Abstract

5-fluoro-2'-deoxyuridine (floxuridine, 5-FdUrd) and 5-fluorouracil (5-FU) are widely used for the treatment of colorectal cancers. The mechanisms of action of 5-FdUrd and 5-FU, as well as the biochemical pathway responsible for their metabolism, are well understood. Identification of every metabolite and achieving mass balance by conventional UV absorption-based HPLC analysis are not feasible because the metabolites beyond 5-FU in the 5-FdUrd metabolic pathway are undetectable by UV light. We therefore established a mass spectrometry method, designed for fast and convenient analysis, for simultaneously measuring 5-FdUrd, 5-FU, and their metabolites. Linearity, precision and accuracy were validated in the concentration ranges studied for each compound. Hydrolysis studies of 5-FdUrd and amino acid mono ester prodrugs of 5-FdUrd in Capan-2 cell homogenates were carried out and the achievement of mass balance was established with this method (recovery of 5'-O-L-leucyl-FdUrd was 96.6 – 108.2% and that of 5-FdUrd was 79.4 – 117.4%). This simple LC-MS method achieves reliable quantitation and mass balance of 5-FdUrd, 5-FU, and their metabolites and can be effectively utilized for further kinetic studies.

Keywords

α -fluoro- β -alanine (FBAL); α -fluoro- β -ureidopropionate (FUPA); 5-fluorodihydrouracil (FUH₂); 5-fluorouracil (5-FU); floxuridine (5-FdUrd); floxuridine prodrug; metabolite; LC-MS; Mass balance

1. INTRODUCTION

5-fluoro-2'-deoxyuridine (floxuridine, 5-FdUrd) and 5-fluorouracil (5-FU) are currently used in the treatment of colorectal cancers. The reversible conversion between 5-FdUrd and 5-FU, their mechanisms of action, and the biochemical pathway leading to subsequent

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metabolite production are all well established [1,2]. The enzymes responsible for their metabolic transformations thus far identified include thymidine phosphorylase (TP), dihydropyrimidine dehydrogenase (DPD), dihydropyrimidinase, and β -ureido-propionase [3–6].

The incorporation of 5-FU into RNA causes toxicity, while the use of 5-FdUrd minimizes adverse effects because of its favored incorporation into DNA [7–9]. Several groups, moreover, have reported greater potency of 5-FdUrd in inhibiting cancer cell proliferation compared to 5-FU [10,11]. It is clear from these studies that protection of the glycosidic bond in 5-FdUrd, such as via the formation of an amino acid prodrug, may lead to higher potency and enhance its therapeutic efficacy. However, the conversion of 5-FdUrd to 5-FU rapidly occurs in many tissues including liver; 5-FU is also decomposed by DPD, which is a rate-limiting enzyme controlling pyrimidine metabolism [12,13]. Therefore, it is important to evaluate the activity of metabolism-related enzymes such as TP and DPD for pyrimidine analogs or the production of catabolites of pyrimidine analogs such as 5-FU and 5-FdUrd.

Several methods have been reported for the identification and quantification of 5-FU analogs, 5-FU and their metabolites [14–19]. However, to our knowledge there have been few studies performing quantitative measurements of all metabolites of 5-FdUrd/5-FU along with a time course of drug-associated kinetic changes and, moreover, achieving mass balance. In this paper, we describe an LC-MS method for the determination of 5-FdUrd, 5-FU, 5-fluorodihydrouracil (FUH₂), α -fluoro- β -ureidopropionate (FUPA), and α -fluoro- β -alanine (FBAL), as well as an amino acid monoester prodrug of floxuridine (5'-O-L-leucyl-FdUrd), and for the quantitation of all metabolites to achieve mass balance.

2. Experimental

2.1 Chemicals and reagents

All chemicals, including 5-fluorouracil (5-FU), 5-dihydrofluorouracil (FUH₂, 5-fluorodihydropyrimidine-2,4-dione), and α -fluoro- β -alanine (FBAL) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO), unless otherwise noted. Floxuridine (5-FdUrd) was obtained from Lancaster Synthesis, Inc. (Windham, NH). α -fluoro- β -ureidopropionate (FUPA) was obtained from Toronto Research Chemicals Inc. (North York, Ontario). Boc-L-leucine was obtained from Chem-Impex (Wood Dale, IL). Cell culture reagents were obtained from Invitrogen (Carlsbad, CA) and cell culture supplies were obtained from Corning (Corning, NY) and Falcon (Lincoln Park, NJ). Methanol was purchased from Burdick & Jackson (Muskegon, MI). All chemicals were either analytical, HPLC, or LC-MS grade.

2.2 Floxuridine prodrug synthesis

The synthesis and characterization of 5-mono amino acid ester prodrugs of 5-FdUrd have been reported previously [5,20]. Briefly, Boc-protected leucine (Boc-L-leucine; 1 mmol), *N,N*-dicyclohexylcarbodiimide (1 mmol), and *N,N*-dimethylaminopyridine (0.1 mmol) were allowed to react with 5-FdUrd (1 mmol) in 7 mL dry dimethylformamide (DMF) for 24 hours. The reaction progress was monitored by TLC (mobile phase, ethyl acetate), and upon completion the reaction mixture was filtered and DMF was removed under vacuum at 40 °C. The residue was extracted with ethyl acetate (30 mL) and washed with water (2 × 20 mL) followed by saturated NaCl (20 mL). The organic layer was dried over MgSO₄ and concentrated under vacuum. The reaction yielded a mixture of 3'-monoester, 5'-monoester, and 3',5'-diester prodrugs of 5-FdUrd, which were resolved and purified using silica gel (230–400 mesh, 1 × 18 cm) column chromatography (dichloromethane/methanol, 20:1). Peak fractions from each product were pooled as three separate samples, dried under vacuum and the Boc group was removed by treating the residues with 1:1 trifluoroacetic

acid (TFA); dichloromethane (5 mL). After 4 hours, the solvent was removed and the residues were reconstituted with water and lyophilized. The TFA salts of amino acid prodrugs of 5-FdUrd were obtained as white fluffy solids. The combined yield of the 3'-monoester, 5'-monoester, and 3',5'-diester prodrugs of 5-FdUrd was 60%. Prodrugs were determined to be 95 – 99% pure and were easily separated from their parent compounds by reverse-phase HPLC. The observed molecular weights of all prodrugs, determined by electrospray ionization mass spectra (ESI-MS) obtained on a Micromass LCT ESI-MS, were found to be consistent with those predicted by their structures. The structural identities of the prodrugs were confirmed using proton nuclear magnetic resonance spectra (¹H-NMR) were obtained in deuterated dimethyl sulfoxide (DMSO-d₆) in a 300 MHz Bruker DPX-300 NMR spectrometer.

2.3 Instrumentation and chromatographic conditions

LC-MS analysis of the drug amounts in hydrolysis studies was performed in triplicate on an LCMS-2010EV (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with an ESI (electrospray ionization) source. The Shimadzu LC-MS system consisted of a Shimadzu LC-20AD pump with an LC-DGU 20A in-line vacuum degassing solvent and an SIL-20HT autosampler with a Hypercarb column (5 μm, 100 × 2.1 mm; Thermo Scientific), and the effluent from the column was routed directly to the ionization source. The system was controlled by Shimadzu LCMSsolution software (version 3) to collect and process data. All samples were run in an aqueous/organic mobile phase at 0.2 mL/min using the gradients shown in Table 1. The additives used for ionizing the analytes (0.1% formic acid or 0.1% ammonium hydroxide; see 2.4.3 below) were omitted from the mobile phase to facilitate simultaneous detection of a positively and negatively charged analytes without having to change mobile phases. The ESI probe was operated with a detector voltage of 1.5kV, CDL temperature of 250 °C, heat block of 200 °C, and nebulizing gas flow of 1.2 mL/min in negative mode for 5-FdUrd and its metabolites, and in positive mode for the floxuridine prodrug. The drying gas was N₂ delivered at 0.1 MPa. The instrument was operated in SIM mode to detect the specific compound ions, and in scan mode to detect the possible fragments for each compound.

2.4 Sample preparation

2.4.1 Tissue culture—The human pancreatic cancer cell line Capan-2 (passages 33–35) from American type Culture Collection (Rockville, MD) was routinely maintained in RPMI-1640 containing 10% fetal bovine serum at 5% CO₂ and 90% relative humidity at 37 °C.

2.4.2 Cell homogenization/hydrolysis sample preparation—Capan-2 cell homogenates were made according to previously reported methods [5]. Briefly, confluent Capan-2 cells were rinsed twice with phosphate-buffered saline (PBS). The cells were resuspended in 5 mL of hypotonic phosphate buffer pH 7.4 (20 mmol/L), lysed by ultrasonication (Micro ultrasonic cell disrupter model KT40, Kontes, Vineland, NJ), centrifuged for 5 min at 1000 × g and the supernatants saved for subsequent assays. Protein amount was quantified with Bio-Rad (Hercules, CA) DC Protein Assay using bovine serum albumin as a standard, and concentration was adjusted to 500 μg/mL with PBS. The lysate supernatant (250 μL) was added to each well of a 96-well plate (Corning), and the reactions were started by the addition of substrate and incubated at 37 °C for 60 min. At the desired time points, sample aliquots (35 μL) were removed and added to 150 μL of acetonitrile containing 0.1% formic acid to stop the hydrolysis reaction, as this solvent condition was previously found to be favorable for the chemical stability of amino acid ester prodrugs and 5-FdUrd [5,20–22].

2.4.3 LC-MS sample preparation—The sample preparation procedure for LC-MS was derived from that previously used by our lab for the detection of 5-FdUrd and its prodrugs by HPLC [5,23,24]. Briefly, the hydrolysis samples from 2.4.2 were filtered through 0.45 μm filters at 1000g for 10 min at 4 °C. The filtrate (100 μL) was diluted in 100 μL of Milli-Q water with either 0.1% formic acid for the detection of 5'-O-L-leucyl-FdUrd, or 0.1% ammonium hydroxide for the detection of 5-FdUrd, 5-FU, FUH₂, FUPA, and FBAL.

2.5 Method validation

2.5.1 Linearity—5-FdUrd, 5-FU, FUH₂, FUPA, FBAL and 5'-O-L-leucyl-FdUrd were dissolved in DMSO at a concentration of 2.5 mg/mL and stored at -20 °C. Calibration curves for each compound were prepared by serially diluting into a mixture of cell lysate and acetonitrile and lastly diluted into Milli-Q water containing 0.1% formic acid for the detection of 5'-O-L-leucyl-FdUrd or 0.1% ammonium hydroxide for the detection of 5-FdUrd, 5-FU, and their metabolites. In addition, intra-day (total assay time) and inter-day (24 hr) chemical stabilities for standard solutions were determined. The final concentrations were 1, 5, 10, 25, 50, 100, 500, 1000 ng/mL for 5'-O-L-leucyl-FdUrd, 50, 100, 500, 1000, 2500 ng/mL for 5-FdUrd, 25, 50, 100, 500, 1000, 2500 ng/mL for 5-FU, 50, 100, 500, 1000, 2500 ng/mL for FUH₂, 25, 50, 100, 500, 1000, 2500 ng/mL for FUPA and 50, 100, 500, 1000, 2500 ng/mL for FBAL. All samples were freshly diluted from the 2.5 mg/mL stocks immediately before the start of each assay; each assay run in this way was one of three replicates. Quality control samples were also prepared by serially diluting into a mixture of cell lysate and acetonitrile and lastly diluted into Milli-Q water containing 0.1% formic acid or 0.1% ammonium hydroxide for each standard curve at the following concentrations: 5'-O-L-leucyl-FdUrd, 5-FdUrd, FUPA and 5-FU, 100 and 500 ng/mL; FUH₂ and FBAL, 500 and 1000 ng/mL.

2.6 Limits of quantitation

The limits of quantitation were determined for 5'-O-L-leucyl-FdUrd, 5-FdUrd, 5-FU, FUH₂, FUPA and FBAL. The compounds were serially diluted from their 2.5 mg/mL stocks into a mixture of cell lysate and acetonitrile, and lastly diluted into Milli-Q water containing 0.1% formic acid or 0.1% ammonium hydroxide to obtain the final concentrations, 10 μL of which were injected for analysis by LC-MS. For each compound, the limit of quantitation was calculated as the minimum detectable amount that produced a relative standard deviation of less than 15%.

2.7 Data analysis

The initial rates of hydrolysis were used to obtain the apparent first-order rate constants and to calculate the compounds' half-lives. The apparent first-order degradation rate constants for 5'-O-L-leucyl-FdUrd and 5-FdUrd at 37 °C were determined by plotting the logarithm of prodrug remaining (C) as a function of time. The slopes of these plots are related to the rate constant, k, and given by

$$k = 2.303 \times \text{slope} (\log C \text{ vs. time}) \quad (1)$$

The degradation half-lives were then calculated by the equation,

$$t_{1/2} = 0.693/k \quad (2)$$

Statistical significance was evaluated with GraphPad Prism v. 3.0 by performing one-way analysis of variance with post-hoc Tukey's test to compare means.

3. Results and discussion

3.1 Liquid chromatography

The gradient conditions, consisting of water and either acetonitrile or acetonitrile/methanol (70:30), were adopted and modified for this analysis from a previous report (Table 1) [17]. 5'-O-L-leucyl-FdUrd, 5-FdUrd, 5-FU, FUH₂, FUPA, and FBAL are all relatively hydrophilic compounds (Table 2). The Hypercarb column, which consists of fully porous spherical carbon particles, is capable of very high resolution of closely related polar molecules and thus was an ideal choice for the analysis of our test compounds. The elution gradients are shown in Table 1 and were performed for a total run time of 23 min. The retention times of FBAL, FUPA, FUH₂, 5-FU, and 5-FdUrd were quite different; under our run conditions, 5'-O-L-leucyl-FdUrd, 5-FdUrd, 5-FU, FUH₂, FUPA, and FBAL eluted at 5.74, 8.43, 3.35, 2.00, 1.60, and 1.50 min, respectively (Table 2). Not surprisingly, the relatively hydrophobic compounds 5-FU, 5-FdUrd and 5'-O-L-leucyl-FdUrd required the highest percentages of organic solvent (80 or 90%) in the mobile phase to be eluted.

3.2 Mass spectrometry

The chemical structures of 5'-O-L-leucyl-FdUrd, 5-FdUrd, 5-FU, FUH₂, FUPA, and FBAL are presented in Figure 1. The optimal mass spectrometry conditions for the detection of fluorinated compounds (pyrimidine analog and its metabolites) and for the detection of amino acid monoester prodrugs of 5-FdUrd were achieved in negative ion mode ($[M-H]^-$) and in positive ion mode ($[M+H]^+$), respectively. The $[M-H]^-$ of 5-FdUrd, 5-FU, FUH₂, FUPA, and FBAL ions were observed at m/z 245.00, 128.90, 130.90, 148.90, and 105.90, respectively, while the $[M+H]^+$ of the floxuridine prodrug 5'-O-L-leucyl-FdUrd ion was observed at m/z 360.10. The analytical mass spectrometry data are summarized in Table 2. The fragmentation of 5-FdUrd corresponded to the cleavage of the glycosidic bond to yield an ion at m/z 128.90. The fragmentation of FUPA corresponded to the cleavage of the peptide bond to yield an ion at m/z 105.90.

3.3 Linearity and quantitation limits

We assessed the chemical stability of the compounds by comparing the slopes of the standard curve samples injected on day 1 with those prepared at the same time and injected 24 hours later. In a separate experiment with 5-bromouracil as the internal standard, the recovery using the sample preparation procedure described in 2.4.2 and 2.4.3 exhibited a range of 0.992 – 1.033 throughout all standard concentrations tested (data not shown). The assay was validated without an internal standard for linearity of the calibration curves by running the following standards, injected in triplicate (freshly prepared before each injection): 5'-O-L-leucyl-FdUrd, 1 – 1000 ng/mL; 5-FdUrd, 50 – 2500 ng/mL; 5-FU, 25 – 2500 ng/mL; FUH₂, 50 – 2500 ng/mL; FUPA, 25 – 2500 ng/mL; FBAL, 50 – 2500 ng/mL. The limits of quantitation for 5'-O-L-leucyl-FdUrd, 5-FdUrd, 5-FU, FUH₂, FUPA, and FBAL were 0.5, 25, 25, 50, 50 and 125 pg, respectively, based on 10 μ L injections of samples serially diluted from concentrated stocks. The linear regression analysis results, without weighting factors, are summarized in Table 3 and expressed as the slopes of the concentration curves. Note that the slopes of these compounds, with the exception of FUH₂ and FBAL, do not significantly decrease in the presence of cell lysate and acetonitrile with either 0.1% formic acid or 0.1% ammonium hydroxide in Milli-Q water at room temperature over 24 hours (Table 3, A vs. B); we conclude that these compounds are therefore chemically stable under these conditions. Conversely, at first glance it would appear that FUH₂ and FBAL have stability issues under these conditions, especially given the relatively

large decreases in their slopes (33.4% and 76.9%, respectively) after 24 hours. However, the stability of these two metabolites over the duration of the assay itself likely does not significantly impact the determination of mass balance for primarily two reasons. First, preliminary data from our lab showed that less than 3% of FUH₂ and less than 6% of FBAL were lost within the time course of the assay. Second, even at their maximum values, the contribution of FUH₂ and FBAL to the total mass calculation for both 5-FdUrd and 5'-O-L-leucyl-FdUrd was still relatively small (Figure 3).

3.4. Precision and accuracy

The precision and accuracy were determined at each sample concentration in triplicate; the accuracy values are presented in Table 4. The average precision varied from 1.0 to 13.1% for 5'-O-L-leucyl-FdUrd, 0.6 to 14.9% for 5-FdUrd, 0.1 to 11.4% for 5-FU, 0.4 to 11.4% for FUH₂, 0.2 to 12.2% for FUPA, and 2.8 to 11.9% for FBAL. The accuracy, which is expressed as the ratio of compound added to that measured, exhibited the following ranges: 5'-O-L-leucyl-FdUrd, 1.1 – 9.3%; 5-FdUrd, 1.0 – 8.7%; 5-FU, 0.1 – 10.5%; FUH₂, 0.3 – 8.3%; for FUPA, 0.7 – 11.9%; FBAL, 0.2 – 10.6%.

3.5 Recovery

The recovery rates were determined with triplicate samples per time point. The recovery result was used to calculate the mass balance of starting compound, either 5'-O-L-leucyl-FdUrd or 5-FdUrd.

Nearly complete recovery of the starting materials was achieved, and the average recovery of the analytes from 5-FdUrd and amino acid monoester prodrugs of 5-FdUrd ranged from 79.4 – 117.4% (Figure 3 and Table 5). Figure 3 shows that 5-FdUrd and 5'-O-L-leucyl-FdUrd exhibited rapid degradation, and approximately 70% of the starting materials were recovered as 5-FU in both hydrolysis studies (Figures 3A and 3B). The standard deviations of recovery % increased at later time points for 5-FdUrd and 5'-O-L-leucyl-FdUrd, ranging from 0.0% to 31.8%. In general, the average recovery declined with time, especially for 5-FdUrd after 60 min (Table 5). It has been reported that further metabolism of FBAL to fluoride ion might reduce the recovery rate [25]. The half-life of 5'-O-L-leucyl-FdUrd (4.8 min) was calculated based on the disappearance of the prodrug; this result is in agreement with our previous report [5].

4. Conclusions

Fluoropyrimidines such as 5-FdUrd and capecitabine currently see fairly widespread use as chemotherapeutic agents. Mass balance studies such as those performed here provide valuable data for determining clinically relevant parameters such as pharmacokinetics and clearance mechanisms/pathways of test drugs. The metabolic pathway that leads from 5-FdUrd to 5-FU and subsequent metabolites has been well characterized [1,5,26,27]. Furthermore, the clinical pharmacokinetics of 5-FU in cancer patients has been thoroughly investigated along with the kinetics of its catabolites [28,29]. However, to our knowledge, all metabolites were not closely monitored and mass balance was not accomplished in those investigations. The achievement of mass balance would provide a basis for a more accurate determination of pharmacokinetic parameters of test drugs, especially when one of the metabolites still exhibits therapeutic effects [30]. Several analytical methods have been developed to quantify those pyrimidine analogs [14,16,17,31,32]; however, many of these methods do not allow for the simultaneous determination of pyrimidine analogs and all of their metabolites. Moreover, none of these procedures in *in vitro* and *in vivo* studies achieved mass balance for 5-FdUrd or 5-FU, or for their prodrugs and metabolites. Therefore, a high-throughput LC-MS methodology was developed in this study for the rapid

and simultaneous quantitative determination of 5-FdUrd, its prodrugs and metabolites. This relatively simple and straightforward method does not require the use of radioactive isotopes. More importantly, mass balance was achieved (recoveries: 5'-O-L-leucyl-FdUrd, 100.0 – 109.0%; 5-FdUrd, 92.0 – 102.6%), which could not be accomplished by conventional LC-UV assays (Figure 3 and Table 5). It has been reported that, at least with some parent drug/metabolite combinations, the use of internal standards for quantitative mass spectrometry can result in quantitative bias due to ion suppression by chemically closely related, co-eluting internal standards [33]. Therefore, it has been suggested that quantitation without an internal standard is in certain cases warranted, even preferable [33]. Indeed, we have found that an internal standard was not required for the quantitation of pyranosides and their metabolites in our *in vitro* experiments, as the recovery yield of all the samples was found to be virtually 100%. However, for 5-fluorouracil and similar chemotherapeutic agents, the inclusion of internal standards such as 5-bromouracil and 5-chlorouracil might still be necessary for accurately quantifying these compounds and their metabolites in biological samples such as blood and urine [34–36]. In summary, the LC-MS methodology described here is accurate, convenient, robust, sensitive, and reproducible for the determination of 5'-O-L-leucyl-FdUrd, 5-FdUrd, 5-FU and their metabolites.

Acknowledgments

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Abbreviations

FBAL	α -fluoro- β -alanine
FUPA	α -fluoro- β -ureidopropionate
FUH₂	5-fluorodihydrouracil
5-FU	5-fluorouracil
5-FdUrd	5-fluoro-2'-deoxyuridine

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Fig. 1.
Metabolic pathway of the floxuridine prodrug 5'-O-L-leucyl-FdUrd (adapted from [1]).

Fig. 2.
Ion chromatograms of (A) FBAL, (B) FUPA, (C) 5-FUH₂, (D) 5-FU and (E) 5-FdUrd over the 23 min run time.

(A) 5-FdUrd

(B) 5'-O-L-leucyl-FdUrd

Fig. 3.

Drug compositions and recoveries of (A) 5-FdUrd and its metabolites and (B) 5'-O-L-leucyl-FdUrd and its metabolites over the 60 min experimental time course. Inset figures show the data for FdUH₂, FdUPA and FBAL rescaled for clarity.

Table 1

Elution gradients for (A) O-L-leucyl-L-FdUrd, and (B) 5-FdUrd and its metabolites

(A)

Time (min)	0	0.5	7	12	15	23
Water (%)	98	98	10	10	98	98
Acetonitrile (%)	2	2	90	90	2	2

(B)

Time (min)	0	2.5	10.5	15	17	23
Water (%)	80	80	20	20	80	80
Acetonitrile: Methanol (70:30) (%)	20	20	80	80	20	20

Table 2
Analytical mass spectrometry data for 5'-O-L-leucyl-FdUrd, 5-FdUrd and their metabolites

Compound	Mode	Retention Time	Observed <i>m/z</i>	Expected <i>m/z</i>	Molecular Weight	cLogP*
5'-O-L-leucyl-FdUrd	[M+H] ⁺	5.74	360.10	360.36	359.36	-0.32
5-FdUrd	[M-H] ⁻	8.43	245.00	245.19	246.19	-1.41
5-FU	[M-H] ⁻	3.45	128.90	129.07	130.07	-0.57
FUH ₂	[M-H] ⁻	2.00	130.90	131.09	132.09	-0.49
FUPA	[M-H] ⁻	1.60	148.90	149.10	150.10	-1.22
FBAL	[M-H] ⁻	1.52	105.90	106.08	107.08	-3.02

5-FdUrd; 5-fluoro-2'-deoxyuridine, 5-FU; 5-fluorouracil, FUH₂; 5-dihydrofluorouracil, FUPA; α-fluoro-β-ureidopropionate, FBAL; α-fluoro-β-alanine.

* cLogP was calculated using ChemDraw Ultra 11.0.

Table 3

Calibration curve parameters (n=3); (A) intra-day, and (B) inter-day

(A)

Parameter	Slope (Mean $\pm$ SD)	Correlation coefficient (Mean $\pm$ SD)
5'-O-L-leucyl-FdUrd	1013.02 $\pm$ 27.72	1.00 $\pm$ 0.00
5-FdUrd	371.75 $\pm$ 2.07	1.00 $\pm$ 0.00
5-FU	240.88 $\pm$ 4.62	1.00 $\pm$ 0.00
FUH ₂	28.44 $\pm$ 0.35	1.00 $\pm$ 0.00
FUPA	212.72 $\pm$ 6.15	1.00 $\pm$ 0.00
FBAL	10.59 $\pm$ 0.41	1.00 $\pm$ 0.00

(B)

Parameter	Slope (Mean $\pm$ SD)	Difference from intra-day (%)
5'-O-L-leucyl-FdUrd	1052.40 $\pm$ 110.33	3.9
5-FdUrd	388.03 $\pm$ 8.56	4.4
5-FU	246.15 $\pm$ 4.61	2.2
FUH ₂	18.94 $\pm$ 1.00	33.4
FUPA	219.37 $\pm$ 28.65	3.1
FBAL	2.44 $\pm$ 0.12	76.9

Table 4

Accuracy of the LC-MS-based detection method

Concentration (ng/mL)	Mean $\pm$ s.d.	Accuracy (%)
5'-O-L-leucyl-FdUrd		
1	1.0 $\pm$ 0.1	1.1
5	5.1 $\pm$ 0.6	1.7
10	10.9 $\pm$ 1.1	9.3
25	26.6 $\pm$ 1.1	6.2
50	53.9 $\pm$ 1.6	7.8
100	106.5 $\pm$ 7.7	6.5
500	475.4 $\pm$ 23.5	4.9
1000	1011.1 $\pm$ 7.8	1.1
5-FdUrd		
50	51.4 $\pm$ 2.7	2.8
100	96.8 $\pm$ 3.0	3.2
500	456.4 $\pm$ 9.1	8.7
1000	956.5 $\pm$ 7.2	4.3
2500	2524.3 $\pm$ 4.3	1.0
5-FU		
25	23.9 $\pm$ 2.4	4.3
50	50.0 $\pm$ 7.4	0.1
100	110.5 $\pm$ 4.1	10.5
500	471.5 $\pm$ 22.6	5.7
1000	1013.8 $\pm$ 16.1	1.4
2500	2497.8 $\pm$ 0.3	0.1
FUH₂		
50	47.6 $\pm$ 4.7	4.8
100	103.9 $\pm$ 9.9	3.9
500	458.7 $\pm$ 15.8	8.3
1000	961.8 $\pm$ 19.6	3.8
2500	2508.7 $\pm$ 13.8	0.3
FUPA		
25	24.6 $\pm$ 2.6	1.8
50	55.9 $\pm$ 5.3	11.9
100	89.1 $\pm$ 22.2	10.9
500	496.6 $\pm$ 66.6	0.7
1000	1058.9 $\pm$ 24.8	5.9
2500	2477.9 $\pm$ 21.6	0.9
FBAL		
50	54.3 $\pm$ 4.0	8.5
100	105.9 $\pm$ 13.7	5.9
500	509.0 $\pm$ 12.5	1.8

Concentration (ng/mL)	Mean $\pm$ s.d.	Accuracy (%)
1000	1106.1 $\pm$ 8.7	10.6
2500	2495.6 $\pm$ 2.6	0.2

Table 5

Recovery percentages of 5'-O-L-leucyl-FdUrd and 5-FdUrd

Time (min)	Drug Compound	
	5'-O-L-leucyl-FdUrd (%)	5-FdUrd (%)
0	100.0 ± 0.0	100.0 ± 0.0
10	97.3 ± 13.9	117.4 ± 16.3
30	108.2 ± 18.8	104.8 ± 21.5
60	96.6 ± 25.0	79.5 ± 26.0

Mean ± SD (n=3)