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Comparison of three derivatization reagents for the simultaneous determination of highly hydrophilic pyrimidine antitumor agents in human plasma by LC–MS/MS

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ABSTRACT

A comparison of three derivatization reagents (dansyl chloride, diazomethane and *p*-bromophenacyl bromide) for the simultaneous quantitation of three anticancer chemicals (tegafur, 5-fluorouracil and gimeracil) and endogenous uracil in plasma using high performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed and evaluated. Through a comprehensive consideration, *p*-bromophenacyl bromide (*p*-BPB) was finally selected as the derivatization reagent. Because it essentially changed the chromatographic behavior of the aforementioned highly hydrophilic compounds and significantly enhanced their sensitivities. The method was validated over the concentration ranges of 5–5000 ng/ml for tegafur, 0.6–700 ng/ml for 5-fluorouracil, 3–700 ng/ml for gimeracil and 6–2000 ng/ml for uracil. The method was successfully applied to the pharmacokinetics study of tegafur, 5-fluorouracil, gimeracil and uracil in cancer patients.

1. Introduction

LC–MS/MS equipped with atmospheric pressure ionization (API) source has proven to be one of the most effective tools in analysis of trace level compounds in complex biological matrices owing to its high sensitivity and selectivity. Along with many successful applications, a number of limitations of API have been reported to analyze some chemicals, which cannot produce free gas ions efficiently or which have low proton affinity [1–4].

S-1 is a new oral fluorouracil antitumor drug, which consists of tegafur (FT), gimeracil (CDHP) and potassium oxonate (Oxo) at a molar ratio of 1:0.4:1. FT is a prodrug of 5-fluorouracil (5-FU). Gimeracil (CDHP) maintains a high plasma level of 5-FU for a prolonged period and increases the plasma level of uracil (Ura) by competitive inhibition of dihydropyrimidine dehydrogenase (DPD). Potassium oxonate can reduce the gastrointestinal toxicity induced by 5-FU [5–9]. The structures of three little antitumor agents of 5-FU, FT, CDHP and endogenous Ura are shown in Fig. 1. It was reported that 5-FU and CDHP could be determined by gas chromatography/mass spectrometry (GC/MS) after derivatizated with pentafluorobenzylbromide, and FT could be determined by HPLC without derivatization. The LLOQ of 5-FU, CDHP and FT reached 1, 2 and 10 ng/ml, respectively [5]. Zhong determined the 5-FU, CDHP and FT by LC–MS/MS without derivatization procedure, by which the LLOQ of FT, 5-FU and CDHP reached 12, 2 and 2 ng/ml, respectively [10]. Due to their high hydrophilicity and low production efficiency of free gas ions in ion sources, the pyrimidine analogues show poor retention in common reversed phase liquid chromatography and display low mass spectrometry response in either electrospray ionization (ESI) source or atmospheric pressure chemical ionization (APCI) source. The methods incorporated with hydrophilic interaction chromatography (HILIC) to improve the retention and MS response of highly hydrophilic compounds and achieved sensitive analysis have been noted [11–14]. However, our study showed that the improvement of the sensitivity was limited. On the other hand, we found that some endogenous impurities might retain in HILIC column, which led to serious matrix effects and interferences.

In order to improve the detection sensitivity of these hydrophilic acidic compounds, many derivatization methods have been developed to enhance their ionization efficiency. *p*-Bromophenacyl bromide (*p*-BPB) is a good derivatization reagent for carboxylic acid and thiol-containing compounds [15–19]. Its analogue 4-bromomethyl-7-methoxycoumarin has been applied to improve

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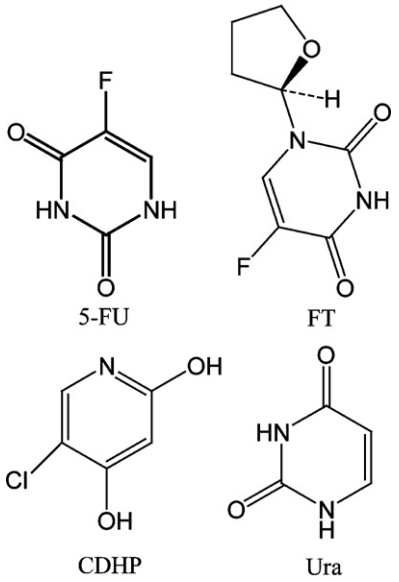

Fig. 1. The structures of 5-FU, FT, CDHP and Ura.

the sensitivity of 5-FU in APCI source successfully [20]. Derivatization with dansyl chloride (DNS-Cl) can improve the sensitivities of steroidal estrogens, cysteine, miazines and phenolic compounds in LC–MS/MS by introducing a functional group with high proton affinity. Its application has been widely reported, and it has also been used for the analysis of 5-FU in human plasma by LC–MS/MS with a lower limit of quantification (LLOQ) of 5 ng/ml [21–26]. Methylation with diazomethane (CH_2N_2) has been mostly used for the determination of acids by GC/MS [27–29].

The aim of this work is to establish an optimal derivatization method for the simultaneous determination of the aforementioned highly hydrophilic pyrimidine analogues by LC–MS/MS. The validated method was successfully applied to a clinical pharmacokinetic study in cancer patients.

2. Experimental

2.1. Materials, reagents and instrumentation

Tegafur, 5-fluorouracil and uracil were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Gimeracil was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). 5-Chlorouracil used as the internal standard (IS) was purchased from J&K Scientific Co., Ltd (Beijing, China). *p*-Bromophenacyl bromide and dansyl chloride were purchased from Sigma–Aldrich (Shanghai, China). Sodium bicarbonate, ammonium acetate, ethyl acetate, formic acid and hydrochloride were purchased from Nanjing Chemical Reagents Co., Ltd (Nanjing, China). Isopropanol was purchased from Jiang Su Hanbon Science & Technology Co., Ltd (Huaian, China). Acetonitrile and methanol were purchased from Merck & Co. Inc (Darmstadt, German). Distilled water was used throughout the study.

The liquid chromatography was performed on an Agilent 1200 series liquid chromatography (Agilent Technologies, Palo Alto, CA, USA), which included an Agilent 1200 binary pump (model G1312B), a vacuum degasser (model G1322A), an Agilent 1200 autosampler (model G1367C) and a temperature controlled column compartment (model G1330B). The LC system was coupled with an Agilent 6410B triple quadrupole mass spectrometer (USA) equipped with an electrospray ion source (model G1956B). The signal acquisition and peak integration were performed using the Masshunter Qualitative Analysis Software (B.03.01Build 346) supplied by Agilent Technologies.

Table 1
Gradient elution program.

	Time (min)							
	0	1	1.1	13	13.2	15.7	15.9	19
Acetonitrile (%)	30	30	45	45	98	98	30	30

2.2. Liquid chromatography/mass spectrometry operating conditions

The chromatographic separation was achieved on a Zorbax SB-Aq column (150 mm × 2.1 mm I.D., 3 μm, Agilent, Wilmington, DE, USA) at 38 °C. The mobile phase for gradient elution consisted of two solvent systems: solvent A, acetonitrile; solvent B, 5 mM ammonium acetate buffer solution containing 0.1% formic acid, and it was delivered at a flow rate of 0.5 ml/min. The details of the gradient elution program are described in Table 1.

The quadrupole mass spectrometer equipped with an ESI source was set with a drying gas (N_2) flow of 12 L/min, nebulizer pressure of 60 psi, drying gas temperature of 350 °C, capillary voltage of 4.0 kV in positive ion mode. The multiple reaction monitoring (MRM) transitions, fragmentor voltage and collision energy for the quantitative experiments are displayed in Table 2.

2.3. Preparations of the standards and quality control (QC) samples

Stock solutions (1 mg/ml) of FT, 5-FU and CDHP were prepared in methanol and stored at −20 °C. The solutions were further diluted with methanol to give a series of standard working solutions with concentrations of 0.1, 1, 10 and 100 μg/ml for FT and CDHP. Similarly, concentrations of 0.01, 0.1, 1, 10 and 100 μg/ml were prepared for 5-FU. Stock solution (1 mg/ml) of Ura was prepared in distilled water and stored at 4 °C. The solution was further diluted with distilled water to give a series of standard working solutions with concentrations of 0.1, 1, 10 and 100 μg/ml for Ura. The calibration standards were freshly prepared by spiking appropriate amount of the standard working solutions into 0.5 ml pooled human plasma. Low, medium and high level QC samples were prepared for FT (10, 800 and 4400 ng/ml), 5-FU (1.5, 60 and 600 ng/ml), CDHP (8, 80 and 600 ng/ml) and Ura (15, 400 and 1800 ng/ml), respectively.

The *p*-BPB reagent was prepared freshly by dissolving 100 mg in 10 ml acetonitrile to give a concentration of 10 mg/ml. The stock solution (1 mg/ml) of the IS was prepared in distilled water and further diluted to 10 μg/ml with distilled water and used for all over the analyses.

2.4. Sample preparation

Sample preparation involved liquid–liquid extraction and derivatization. Aliquot of 500 μl plasma and aliquot of 30 μl of the IS

Table 2
The MRM transitions, fragmentor voltage and collision energy for quantitative analysis of FT, CDHP, 5-FU and Ura.

	m/z precursor ion	*m/z* product ion	Fragmentor voltage (V)	Collision energy (V)
FT	397.0	327.0	65	4
CDHP	539.9	168.9	215	40
5-FU	524.9	310.0	155	24
Ura	506.9	118.0	150	50
CU	540.9	327.9	160	24

(10 μg/ml) were both placed into a 10 ml glass centrifuge tube, then 50 μl of 1 M hydrochloric acid was added into it and vortexed for 30 s. After the addition of 3 ml of isopropanol–ethyl acetate (15:85, v/v), the mixture was vortex-mixed for 5 min and centrifuged at 2685 × *g* for 10 min. The organic layer was transferred and followed by spiking 50 μl of saturated sodium bicarbonate solution, and the mixture was evaporated to dryness at 40 °C.

All procedures mentioned below were performed under non-actinic light. 50 μl of saturated sodium bicarbonate solution and 300 μl of *p*-BPB (10 mg/ml) acetonitrile solution were added to dissolve the residue and vortex-mixed, then the tubes were sealed and placed in a water bath at 60 °C for 30 min. After extraction of the derivatives by 3 ml of ethyl acetate, 300 μl of distilled water was added and vortexed to remove the salts and some impurities. Following centrifugation and separation, the organic phase was transferred and evaporated to dryness at 40 °C. The residue was reconstituted with 100 μl of acetonitrile and centrifuged at 17,000 × *g* for 5 min, the supernatant was transferred into a sealed injection vial for LC–MS/MS analysis.

2.5. Application in pharmacokinetics study

The method described above was applied to the pharmacokinetic study of S-1 capsule in twelve advanced gastric cancer patients after single administration. The dose of S-1 was determined according to their body surface area (BSA) as follows: for BSA ≤ 1.25 m^2, 40 mg; for BSA > 1.25 m^2, 60 mg. Blood samples (5 ml) were drawn before intake and at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 14, 24, 48, and 60 h after oral administration. The samples were collected into heparinized tubes and were immediately centrifuged at 2685 × *g* for 15 min at 5 °C. The plasma obtained was frozen at −80 °C until analysis.

3. Results and discussion

3.1. Method selection

Initially, several attempts were carried out in order to determine the analytes directly. The poor retentions of 5-FU and Ura were shown in the C18 column and the low mass spectrometry responses were shown under the condition of the corresponding mobile phase. Therefore, we tried the HILIC Polar-100 column (150 mm × 2.1 mm I.D., 5 μm, Sepax, Newark, DE, USA) and Amino column (150 mm × 2.1 mm I.D., 5 μm, Hanbon, Huaian, Jiangsu, China), but the retention and the sensitivity were not improved satisfactorily. Then the sample preparation procedure was also investigated for improving the sensitivity of the method. However, low recoveries were shown when using the solid phase extraction with Waters OASIS HLB Cartridge, Waters OASIS MAX Cartridge or Agela Cleanert PSA Cartridge, and the optimal liquid–liquid extraction method could not meet the requirement of the assay, either. Finally, a derivatization procedure was carried out in order to decrease the hydrophilicity of the analytes and enhance their production efficiency of free gas ions in ion sources.

3.2. Selection of derivatization reagents

DNS-Cl was investigated firstly for its high proton affinity. The experimental results suggested that Ura, 5-FU, CDHP and FT had all reacted with DNS-Cl in a basic aqueous medium successfully. However, there were two kinds of dansyl derivatives, the mono- and bis-dansyl forms, in the final dansyl products for 5-FU and Ura, and the amount ratio of the two derivatives in the final products was not constant. In addition, the derivative yield of FT was only about 10%. The reason might be that the dansylation on the weaker active site was not stable enough, thus the formation and decomposition of dansyl products would proceed simultaneously in the basic aqueous medium. The hydrolysis of the dansyl-5-FU and dansyl-Ura in basic aqueous medium was investigated. The results showed that parts of bis-dansyl products were transformed into mono-dansyl products, the higher pH condition could accelerate this transformation, and the mono-dansyl products could be further hydrolyzed. Then the nonaqueous medium was investigated to avoid the hydrolysis of the derivative analytes. Acetonitrile served as the reaction medium, potassium carbonate was added to supply the basic condition and 18-crown-6 was used to increase the solubility of potassium carbonate by complexing with the potassium ion in solution [20]. The experimental results showed that 5-FU, Ura and CDHP could be transformed into their bis-dansyl derivatives successfully, and the FT could be transformed into its mono-dansyl derivative completely. However, we observed the formation of low yields of dansyl-CDHP and dansyl-Ura due to their poor solubility in basic acetonitrile and to the matrix effect caused by the residual 18-crown-6.

The limits of detection (LODs) were determined for concentrations possessing a signal-to-noise ratio of 3. Except for the endogenous uracil, the LODs of 5-FU, CDHP and FT, obtained after derivatization with DNS-Cl in basic aqueous medium for a 0.5 ml plasma aliquot, were achieved with the following values 0.05 ng/ml, 0.3 ng/ml and 2 ng/ml, respectively. Although high sensitivity was obtained after dansylation, the diversity of the products made it to be non reproducibility and hard to achieve, thus its application for the determination of the pyrimidine analogues was limited.

Methylation with diazomethane (CH_2N_2) could reduce the hydrophilicity of highly polarized compounds and prolonged their chromatographic retention times in reversed liquid chromatography. The advantages of diazomethane are its intensive reactivity with acidic analytes, and its high volatility allowing facile removal of excess reagent [30,31]. In our experiments diazomethane (CH_2N_2) derivatization was performed in a two-phase (water-diethyl ether) system. The derivatives could be easily dissolved in organic phase in the two-phase system, thus their hydrolysis could be avoided. On the other hand, the reaction procedure actually consisted of two functions: one was derivatization, and the other was purification.

The optimal derivatization procedure was as follows: 2 ml of diazomethane diethyl ether solution was added to 0.5 ml of spiked plasma and vortexed for 20 min using a vortex mixer. Diazomethane reagent (diazomethane in diethyl ether) was prepared according to the Aldrich Technical Bulletin AL-180 [32]. The experimental results showed that CDHP, 5-FU and Ura were all converted into their bis-methyl ester products completely, and FT was converted into its mono-methyl ester product completely. However, the LODs of 5-FU, CDHP and FT were only 5 ng/ml, 5 ng/ml and 10 ng/ml, respectively, for a 0.5 ml plasma aliquot. The reason could be that the reduction of the hydrophilicity for the analytes after methylation was limited.

The derivatization of Ura, 5-FU, CDHP and FT with *p*-BPB were investigated in basic aqueous medium. The experimental results showed that FT, which had only one active site, was completely changed into its mono-*p*-BPB form. CDHP, 5-FU, Ura and the IS, which had two reactive sites, were completely transformed into their bis-*p*-BPB forms in their products. The yields of the derivatization all reached 98%. The product ion scan mass spectra of FT, 5-FU, CDHP, Ura and the IS derivatized with *p*-BPB are presented in Fig. 2. The LODs of 5-FU, CDHP and FT were achieved 0.2 ng/ml, 1 ng/ml and 0.3 ng/ml, respectively, for a 0.5 ml plasma aliquot. Derivatization with *p*-BPB not only changed the chromatographic behavior of highly hydrophilic compounds, but also significantly enhanced their MS responses. Also, the high purity of the derivative products and their high yields ensured the good reproducibility. In our

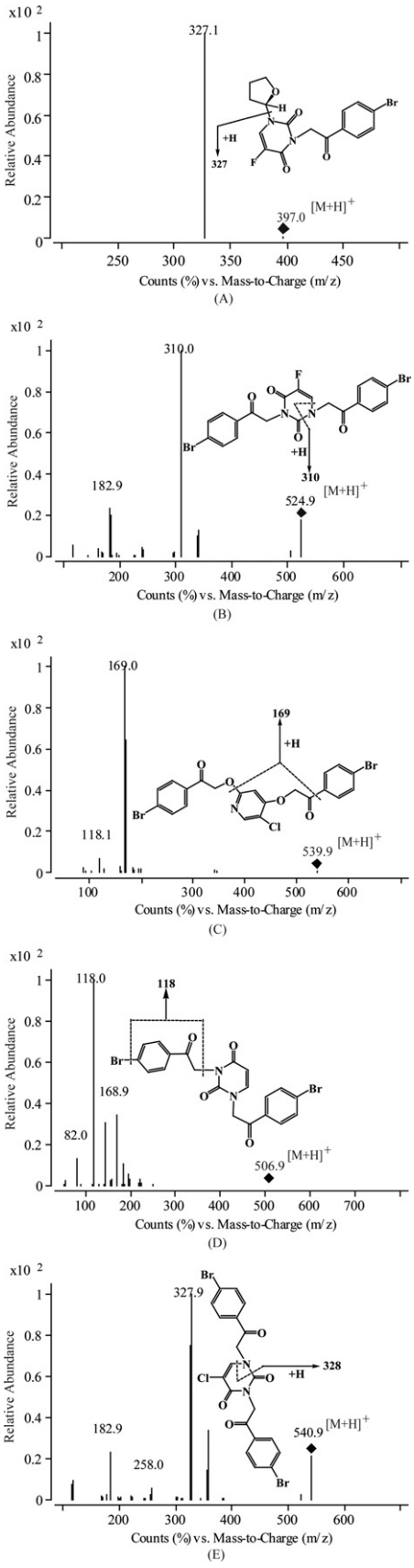

Fig. 2. The product ion scan mass spectra of FT (A), 5-FU (B), CDHP (C), Ura (D) and the IS (E) derivatized with *p*-BPB.

experiments, the derivatization reagents 2, 4-dinitrochlorobenzene (CDNB) and 9-fluorenylmethylchloroformate (FOMC-Cl) were also investigated. But the results showed that the aforementioned antitumor agents could not react with CDNB, and the derivatization with FOMC-Cl was similar to that of DNS-Cl. Therefore, after a comprehensive consideration, *p*-BPB was selected as the derivatization reagent and more detail derivatization conditions were further explored.

3.3. Optimization of derivatization conditions

p-BPB has good solubility in organic solvent, such as acetonitrile, methanol and acetone. In our experiments, the results showed that when the protic solvent methanol was used, *p*-BPB was mostly transformed into its methyl ester product, and when acetone was used, the reproducibility was poor for its high volatility. Acetonitrile is an unreactive aprotic solvent and has lower volatility than acetone. High yield and good reproducibility were shown in our experiments when acetonitrile was used as the solvent for *p*-BPB.

Other custom effects of the buffer pH, the concentration of derivatization reagent, the volume ratio of the derivatization reagent to buffer, reaction temperature and reaction time were also investigated in this study. The experimental results are displayed in Fig. 3.

3.4. LC conditions

Good separation and sharp peak were achieved in a Zorbax SB-Aq column. A gradient elution program was developed to achieve good separation for the derivative analytes and eliminate the matrix effect. The first step at 30% acetonitrile was set to obtain sharp peak of FT, then 45% acetonitrile was aimed to separate Ura, 5-FU, CDHP and the IS distinctly. The increment to 98% acetonitrile could wash away the strongly retained impurities in column and consequently, eliminate the matrix effect to the next analysis. No significant interference and matrix effect was observed under this condition.

3.5. Sample preparation

Liquid–liquid extraction with isopropanol–ethyl acetate has been reported to achieve high recovery of 5-FU and other polar compounds [33,34]. In our experiments, CDHP could be extracted out from water with ethyl acetate and isopropanol, but it could not be extracted out from plasma. Besides, the recoveries of 5-FU and Ura in plasma were lower than those in water. This result indicated that CDHP, 5-FU and Ura had strong plasma protein binding abilities in plasma. Therefore, in our study, liquid–liquid extraction with isopropanol–ethyl acetate after spiked with hydrochloric acid or ammonium sulfate was investigated. The results showed that the acidic condition could release the plasma protein binding compounds much better. On the other hand, the acidic condition could enhance the dissolubility of the acidic hydrophilic compounds in the organic reagent, thus further improved their recoveries.

Some amount of hydrochloric acid could be extracted out by the ethyl acetate–isopropanol solution, and this appeared to interfere with the derivatization. The reproducibility was poor without treatment of the residual hydrochloride acid in our previous study. A higher temperature of water bath could accelerate the removal of hydrochloride acid when drying the supernatant, but the precision was not satisfied. The addition of saturated sodium bicarbonate into the supernatant before derivatization could completely neutralize the residual hydrochloric acid.

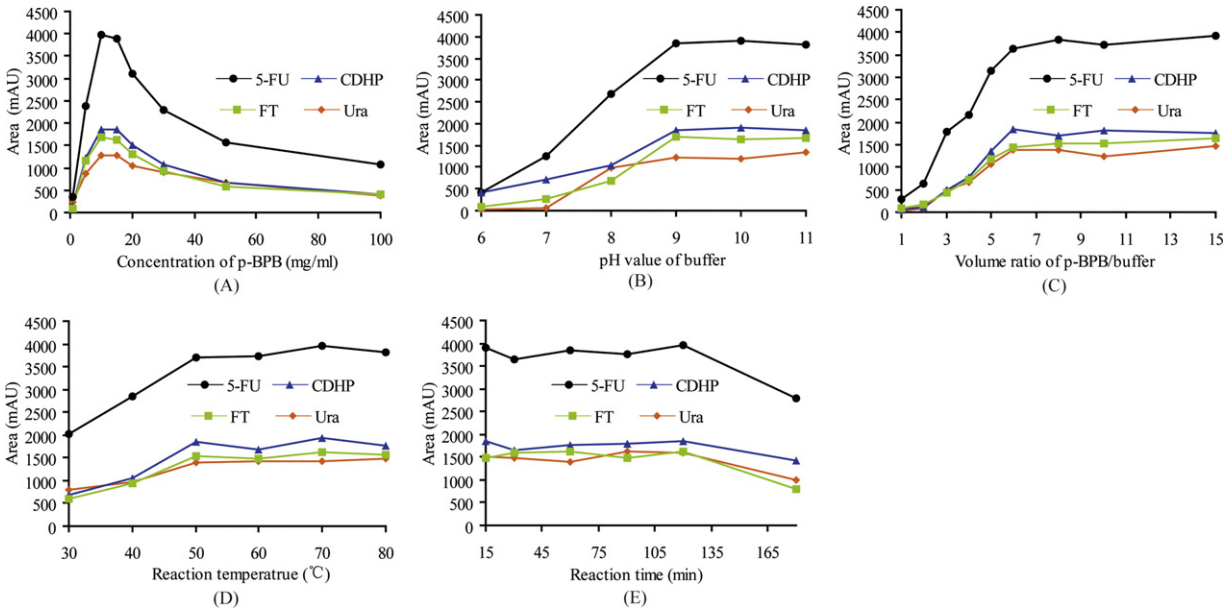

Fig. 3. Effects of concentration of *p*-BPB acetonitrile solution (A), pH value of buffer (B), volume ratio of *p*-BPB/buffer (C), reaction temperature (D) and reaction time (E) on the reactions of *p*-BPB with FT, CDHP, 5-FU and Ura.

3.6. Stability of the derivatives

The *p*-BPB derivatives would degrade rapidly in extremely acidic or basic aqueous solution, but the weak basic buffer-acetonitrile medium and the initial RPLC acidic mobile phase composition could make them stable in one week, and when they were dissolved in acetonitrile, one-month stability could be ensured.

3.7. Method validation

Fig. 4 shows the typical chromatograms of blank plasma (A), LLOQ for FT, CDHP, 5-FU and Ura in plasma and the IS (B), plasma spiked with FT, CDHP, 5-FU and Ura at 5000, 700, 700, 2000 ng/ml, respectively, and the IS (C), and plasma obtained from a gastric cancer patient at 3 h after oral administration of S-1 capsule (D). Typical retention times for FT, CDHP, 5-FU, Ura and the IS were 5.2, 10.3, 9.8, 8.0 and 11.7 min, respectively. No significant interference from endogenous substances was observed at the retention times of the analytes and IS. For endogenous Ura, the response in the blank sample was less than LLOQ sample.

The calibration curves proved to be accurate over the concentration range of 5–5000 ng/ml for FT, 0.6–700 ng/ml for 5-FU, 3–700 ng/ml for CDHP and 6–2000 ng/ml for Ura. The typical linear regression equations were as follows:

$$\text{5-FU}: \quad f = 0.0002611 + 0.002242 \times C, \quad r = 0.9989$$

$$\text{CDHP}: \quad f = -0.0002465 + 0.0005045 \times C, \quad r = 0.9975$$

$$\text{Ura}: \quad \Delta f = 0.002980 + 0.001187 \times C, \quad r = 0.9975$$

$$\text{FT}: \quad f = 0.003306 + 0.001073 \times C, \quad r = 0.9972\,(5\text{–}600\ \text{ng/ml});$$
$$f = 0.3269 + 0.0005589 \times C, \quad r = 0.9975\,(600\text{–}5000\ \text{ng/ml})$$

where f represents the peak area ratio of each analyte to the IS, Δf represents the difference of the peak-area ratio of Ura to the IS in spiked plasma samples and blank plasma samples, and C represents the spiked plasma concentration of analytes. The lower limit of quantification (LLOQ) is the lowest concentration on the calibration curve that can be measured with acceptable accuracy and precision. The LLOQ was evaluated by analyzing samples prepared in five replicates. The measured values of precision and accuracy at LLOQ should be within ±20% [35]. The LLOQ for Ura, which is present in plasma endogenously, was defined as the lowest increased Ura concentration in the blank plasma that can be measured with acceptable accuracy and precision. The mean measured value increased should be within ±20% of the theoretical increased value. The precision around this mean value should not exceed 20% of the CV. The LLOQ for FT, 5-FU, CDHP and Ura were found to be 5, 0.6, 3 and 6 ng/ml in plasma, respectively. The precision and accuracy at this concentration level were acceptable, with RSD values below 10.2% for each analyte.

To study the extraction recovery and matrix effect (ME), the derivative analytes were prepared by spiking the appropriate amount of the standard working solutions, which equal to the three concentration levels of QC samples (five samples each) respectively, into the glass centrifuge tubes, and then derivatizing and drying.

The extraction recovery was estimated by comparing the peak areas of the regularly pretreated QC samples at three concentration levels with those of the derivative analytes reconstituted with 100 μl of acetonitrile. The extraction recoveries ($n = 5$) at three concentration levels of low, middle and high were 80.0%, 78.5% and 82.3% for FT, 81.3%, 81.9% and 73.8% for CDHP, 86.3%, 83.0% and 77.9% for 5-FU and 78.1%, 71.7% and 81.7% for Ura, respectively. The extraction recovery of the IS was 88.7%.

The ME was evaluated by comparing the peak areas of the derivative analytes reconstituted with the blank sample (the final solution of blank plasma originated from five different donors after extraction, derivatization and reconstitution), with those of the derivative analytes reconstituted with 100 μl of acetonitrile. The ME value of the IS was also evaluated. The ME at every concentration level should be less than 15%. The ME ($n = 5$) at three concentration levels of low, middle and high were (93.3 ± 4.9)%, (97.1 ± 3.0)% and (95.0 ± 3.5)% for FT, (109.0 ± 3.8)%, (101.2 ± 4.5)% and (100.2 ± 8.5)% for CDHP, (101.8 ± 3.8)%, (104.2 ± 4.1)% and (102.8 ± 6.2)% for 5-FU and (87.0 ± 2.5)%, (92.4 ± 2.8)% and (94.2 ± 6.3)% for Ura, respectively. The ME of the IS was (100.5 ± 5.1)%. The results showed that there was no matrix effect of the analytes and IS observed in this study.

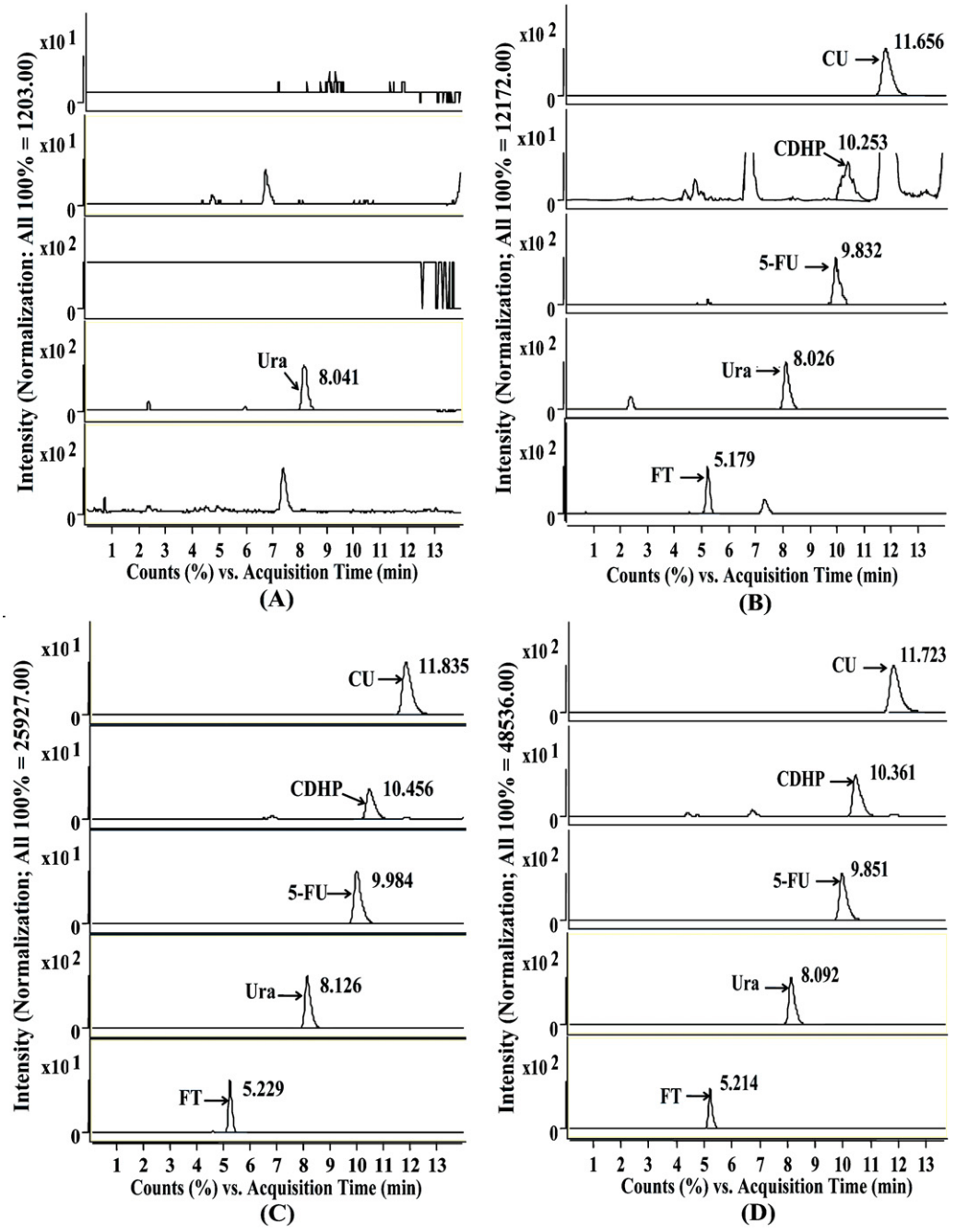

Fig. 4. Typical chromatograms of blank plasma (A), LLOQ for FT, CDHP, 5-FU and Ura in plasma and the IS (B), plasma spiked with FT, CDHP, 5-FU and Ura at 5000, 700, 700, 2000 ng/ml, respectively, and the IS (C), and plasma obtained from a gastric cancer patient at 3 h after oral administration of S-1 capsule (D).

The results for intra- and inter-batch precision and accuracy of the method for the analytes are summarized in Table 3. The precision deviation values for intra- and inter-batch were all within 15% of the RSD at each QC level. All accuracy deviation values for intra- and inter-batch were within 100 ± 15% of the actual values at each QC level. The results revealed good precision and accuracy.

The stability of each analyte in blank human plasma was assessed by analyzing three concentration levels of QC samples at different conditions (time and temperature). The experimental results showed that the analytes were stable in plasma stored at room temperature for 12 h (RE in the range of −5.6% to 8.0% for each analyte), in plasma after three freeze–thaw cycles at −80 °C (RE in the range of −11.2% to 5.9% for each analyte), in plasma stored at −80 °C for 2 months (RE in the range of −10.0% to 6.5% for each analyte) and in plasma stored at −80 °C for 6 months (RE in the range of −10.3% to 5.3% for each analyte).

3.8. Application in pharmacokinetics study

The validated method was successfully applied to quantify FT, 5-FU, CDHP and Ura concentrations in a pharmacokinetic study. Fig. 5 shows the mean plasma concentration–time profiles of each substance in twelve patients with advanced gastric cancer after single administration of S-1 capsule. The maximum concentrations of FT, 5-FU, CDHP and Ura were found to be 2207 ± 545.0 ng/ml,

Table 3
Precision and accuracy of the assay for the determination of FT, 5-FU, CDHP and Ura in human plasma ($n = 3$ batches, five replicates per batch).

Analytes	Concentration (ng/ml)		Precision (%RSD)		Accuracy (%RE)
	Added	Found	Inter-batch	Intra-batch	
FT	10.20	9.900	5.9	8.2	−2.9
	816.0	781.4	7.6	3.6	−4.2
	4488	4515	5.5	4.3	0.6
5-FU	1.521	1.540	5.4	13.4	1.3
	60.84	58.76	7.0	2.3	−3.4
	608.4	613.7	5.8	5.8	0.9
CDHP	8.080	7.810	5.0	6.4	−3.3
	80.80	79.69	8.4	8.2	−1.4
	606.0	586.2	7.0	7.7	−3.3
Ura	15.26	16.26	7.4	9.3	6.6
	406.8	418.6	9.0	5.5	2.9
	1831	1851	5.5	2.4	1.1

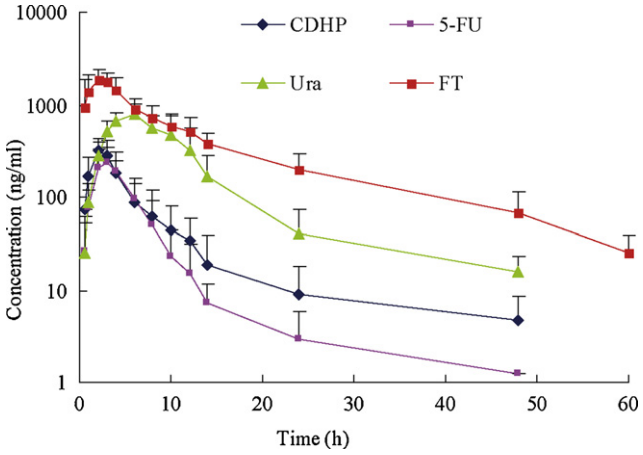

Fig. 5. Mean plasma concentration–time profiles of FT, CDHP, 5-FU and Ura after single administration of S-1 capsule to 12 advanced gastric cancer patients.

245.0 ± 119.1 ng/ml, 374.9 ± 103.0 ng/ml and 827.7 ± 203.4 ng/ml, respectively.

4. Conclusion

Derivatization can decrease the hydrophilicity of highly hydrophilic analytes, enhance their ionization efficiency in ESI source, and thus improve their sensitivity. Furthermore, derivatization with the reagent which has high proton affinity can further enhance their sensitivity. However, the sensitivity is not the only key factor in the choice of derivatization reagent. In contrast with the sensitivity, the reproducibility can be a more important factor.

In this study, after derivatization, the polarities of the analytes were significantly reduced by the less polar or no-polar chemical groups introduced into the molecules of their derivatives. The decrease in the polarity of the derivatives could bring about several advantages for the LC–MS/MS analysis. Firstly, after the active hydrogens in the highly polar small molecular analytes replaced by the alkyl, ester or aryl groups, the efficiency of intermolecular hydrogen bond formation was decreased, thus the tendency of the undesired cluster ion formation in ESI processes was decreased, the efficiency of direct ion evaporation into the gas phase was increased, and thus the amount of the single molecular ions of the analytes in the vapor phase were increased. This improved the ionization efficiency for the analytes. Secondly, by the polarity decreasing of the derivatives, their retentions on the C18 column were increased, and the higher percentage of the organic portion of the mobile phase could be used. By the ratio of the organic portion of the mobile phase increased, the evaporation of the mobile phase in the ESI source became easier, thus the ionization efficiency of the derivatives was improved. In addition, the derivatization provided characteristic fragmentation patterns and intense specific fragment ions with ESI-MS/MS for the trace quantitative analyses.

A sensitive and specific method for simultaneous quantitation of four highly hydrophilic pyrimidine antitumor agents in human plasma by LC–MS/MS after derivatization with *p*-BPB was established. No significant interferences caused by additives and endogenous substances were observed. The method is suitable for the pharmacokinetic study of FT, 5-FU, CDHP and Ura in human. Furthermore, it provides a new strategy to determine other hydrophilic compounds, pyrimidine analogues, acidic compounds and multiply active sites compounds in biological samples.

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