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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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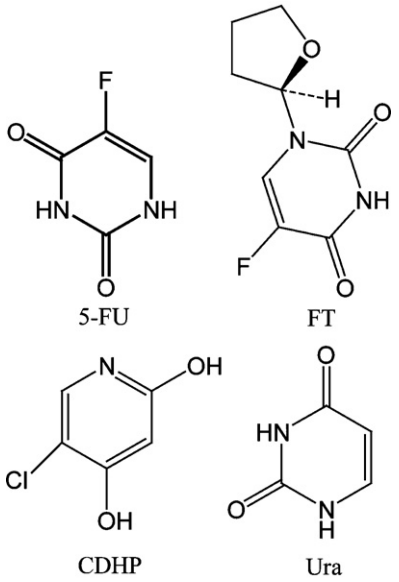

Fig. 1. The structures of 5-FU, FT, CDHP and Ura.

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