Analysis of Three Anticancer Drugs (5-Fluorouracil, Cyclophosphamide and Hydroxyurea) in Water Samples by HPLC-MS/MS

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***Abstract*—**Anticancer drugs are very harmful chemical which use for cancer patient. It has a lot of side effect to cancer patient or anyone who consume contaminated body intake. A rapid, reliable and highly selective performance analysis method was developed for commonly used anticancer drugs (5-Fluorouracil, Cyclophosphamide and Hydroxyurea) residual in water samples. The analytical processes were performed using solid-phase extraction (Oasis@ HLB cartridge) and measured by High performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS/MS). Separation system consist with guard column Agilent@ Zorbax Eclipse XDB C18 (I.D. 4.6 x 50 mm, 1.8 µm particle size) and analytical column Agilent@ Zorbax Eclipse Plus C18 (I.D. 2.1 x 100 mm, 1.8 µm particle size) using gradient mixture of methanol + 0.1% formic acid and water + 0.1% formic acid as mobile phase with multiple injection mode. Simultaneous anticancer drugs were detected by MS/MS using electrospray ionization and multiple reaction monitoring (MRM) for both positive (Cyclophosphamide and Hydroxyurea) and negative (5-Fluorouracil) charges. The method validations were included acceptable, accuracy, precision and specificity for detection of 5-Fluorouracil, Cyclophosphamide and Hydroxyurea shown linearity was achieved from 1 to 50 µg/L, $R^2 > 0.99$ and the calculated limit of detected for 5-Fluorouracil 0.013 µg/L, for Cyclophosphamide was 0.006 µg/L, for Hydroxyurea was 0.050 µg/L. The results from various water sample type were compared to predicted environmental concentrations (PECs) for environmental and human health risk assessment.

***Keywords*—**5-Fluorouracil, Cyclophosphamide, Hydroxyurea, HPLC-MS/MS, water sample.

I. INTRODUCTION

ANTICANCER or antineoplastic drugs refers to any drugs used in chemotherapy of oncological patients. These drugs act by interfering directly of tumour cells and growth cells but acting non-selective and healthy cells may also be damaged which, cause side effects several organisms [1-2].

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Some antineoplastic drugs have already been classified by International Agency for Research on Cancer (IARC) as carcinogens in humans: group 1 such as, cyclophosphamide [3]. Most commonly used of anticancer drugs for the cancer treatment are 5-fluorouracil (5-FU), cyclophosphamide (CP) and hydroxyurea (HU) which, are an antimetabolite, alkylating and other of antineoplastic agents respectively [4] (Fig.1). The contamination routes of these drugs reach to water in the environment by excreted of urine or feces and released via the hospital or domestic wastewater and wastewater treatment plants (WWTPs) [5-7]. Contamination level of pharmaceuticals and personal care products as pollutants (PPCPs) in water samples are importance for environmental and human risk assessment meanwhile, recently considered as emerging environmental contaminants [7]. The aquatic environmental and human health impact of anticancer drugs were imprecised although, there are highly cytotoxic, carcinogenic, embryotoxic, mutagenic and teratogenic [1], [8-9].
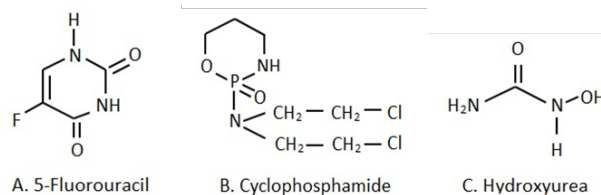

Fig. 1 Structures of most commonly use anticancer drugs

Currently, has not been report for analysis of HU in water samples and other researches were limit or difficulty for analytical method of 5-FU and CP in water samples. Many researchers had been reported by asynchronous drugs and its derivatives while the treatment process possibly use of those three drugs simultaneously [5], [10-20]. Thus, the excretion from cancer patients may contain the residual of those three anticancer drugs with different portion. HPLC-MS/MS as the highly solution performance for qualitative and quantitative analysis, this instrument has been developed and used widely for extensive clinical and environmental studies. Hence, HPLC-MS/MS technique could be developed for

measurements of 5-FU, CP and HU in agents environmental sample, since the simultaneous measurement of 5-FU, CP and HU has not been yet establishes.

The aims of this studier are modifier and developer HPLC-MS/MS method for the simultaneously quantification of 5-FU, CP and HU in water samples. This validated method will be used for measurement the residual of those drugs in various sources of water samples from surface water and domestic wastewater effluent. The contamination in various water samples will be compared to predicted environmental concentrations (PECs) by calculating [21-22], based on consumption data in Thailand.

II. MATERIALS AND METHOD

A. Analytical Method Modification

Analytical method modifications will be done by modified from the single drug analysis of previous researches [10-20]. Advantage and disadvantage of each one will be considered and applying for the simultaneous measurement of those three drugs, such as the mobile phase, gradient and column. Furthermore, those three anticancer drugs have both positive and negative charges for detection which is most difficulty for setting up the analytical procedure with the single run.

B. Chemicals and Reagents

5-Fluorouracil (HPLC-Grade, ≥99%), cyclophosphamide (HPLC-Grade, ≥98%) and hydroxyurea (HPLC-Grade, ≥98%) were purchased from Sigma-Aldrich (USA). Methanol (HPLC-grade, >99.99%) and formic acid (HPLC-Grade, 98-100%) were purchased from Merck (Germany) and ammonia (30%) were purchased from Panreac (Spain).

A stock mixed standard solution of 5-FU, CP and HU were prepared at a concentration of 5 mg/L by dissolving 0.125 mg of the chemicals standard in 25 mL methanol. The mixed standard solution of 5-FU, CP and HU were conducted to prepare the calibration standard. Calibration standard was prepared at a concentration range of 1 to 50 µg/L by dilution of the mixed standard solutions with methanol. All standards and fortification solutions were stored in polypropylene bottle and kept in refrigerator at 4 °C.

C. Instrumentation

All qualification and quantification were performed using an Agilent 1200 SL HPLC coupling with Agilent 6410 triple quadrupole mass spectrometer.

D. Sample Collection and Preparation

Samples of surface water were collected from Chao Phraya River and domestic wastewater effluent from accommodation for cancer patient by grab samples. All the samples were collected in 9-10 and 30 November 2013, respectively and were analyzed within 48 hours. Water samples were collected in polyethylene terephthalate (PET) bottle previously washed with Milli-Q water and methanol before using and rinse by water sample. The collected sample were avoid to sunlight and sent to laboratory, stored in refrigerator at 4°C until analysis.

The methods for analyses the concentration of 5-FU, CP and HU in water samples consisted by using the solid phase extraction (SPE) method coupling with HPLC-MS/MS for quantification [7], [10]. The analysis procedure were shown in Fig. 2
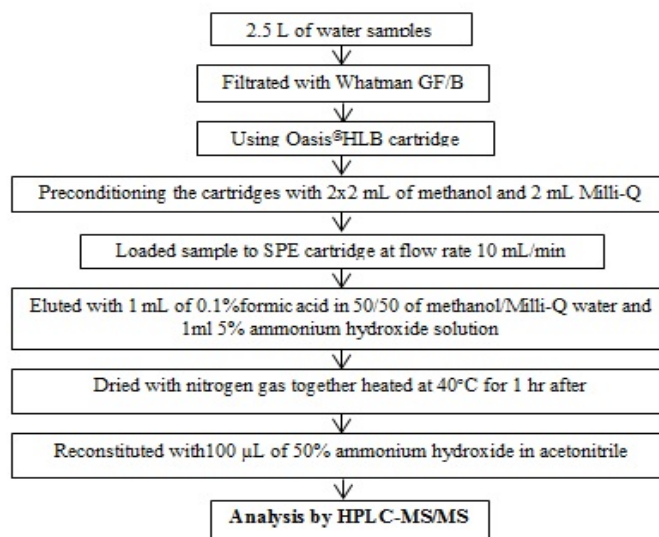

Fig. 2 Water sample analytical procedure

E. Validation of the HPLC-MS/MS Method

The linearity of the experiment will be obtained by using concentration levels of 1, 5, 10, 25 and 50 µg/L with 5 replicates. Acceptance criteria were acceptable accuracy and precision data. The reproducibility and recovery will be obtained by using low and high concentrations relative to calibration range which prepared by using standard of 25 and 50 µg/L spiking 50 mL to 2.5 L of surface water samples with 5 replicates per sample. Calculation of percent recovery as of response in extracted samples compared to control samples and calculation of respective value of relative standard deviation)RSD(. Acceptance criteria were acceptable sensitivity and reproducible recovery.

Estimated of limit of detection)LOD(and limit of Quantitation)LOQ(will be calculated by signal to noise ratio)S/N(. LOD and LOQ were expressed as S/N equal 3 and 10, respectively. The model for calculation of LOD = 3 x S/N and LOQ = 10 x S/N [23].

F. Predicted Environmental Concentrations (PECs)

The preliminary exposure assessment of 5-FU, CP and HU contaminant in surface water will be implemented by calculating PECs [21-22] using the following this parameters model and compared to measurement environmental concentrations in surface water at Chao Phraya river, Bangkok.

$$\text{PECs (mg/L)} = \frac{\text{amount of consumption (mg/year) x excretion fraction of agent x emission of agent to surface water}}{\text{wastewater/person/day(L) x number of people x 365 day x Dilution to surface water}} \qquad (1)$$

where consumption is the quantity of an active molecule consumed by patients and data were collected in 2012 at hospital in Bangkok. The total amounts of 5-FU, CP and HU calculated from usage drugs data in 22 hospitals in Bangkok which about 55% of total hospitals for cancer treatment (Table I). The excretion fraction of 5-FU, CP and HU are 0.20, 0.25 and 0.50 respectively. The fraction of emission of the drug from WWTPs directed to surface water (=1). The volume of wastewater per person per day (default value = 250).

In Bangkok have 7 million people. 365 is the number of days per year (day/year). The dilution factor from wastewater treatment plant (WWTP) effluents to surface waters (default value set at 140).

TABLE I
CONSUMPTION DATA OF 5-FU, CP AND HU IN BANGKOK

Anticancer drug	Total amounts (µg)
5-FU	259,068
CP	118,124
HU	158,026

TABLE II
HPLC-MSMS INSTRUMENTATIONS AND THEIR OPTIMIZED CONDITIONS DEVELOPMENT FOR QUANTIFICATION OF 5-FU, CP AND HU

HPLC		MS	
Instrument	Agilent 1200 SL HPLC	Instrument	Agilent 6410 triple quadrupole mass spectrometer
Column	Guard column Agilent$^@$ Zorbax Eclipse XDB C$_{18}$ (I.D. 4.6x50 mm, 1.8 µm) Analytical column Agilent$^@$ Zorbax Eclipse Plus XDB C$_{18}$ (I.D. 2.1x100 mm, 1.8 µm)	Ionization Polarity mode	Electrospray ionization Negative for 5-FU Positive for CP, HU
Mobile Phase	A: Milli-Q water+0.1% formic acid B: methanol+0.1% formic acid	Nebulizer Gas flow	N$_2$ (50 psi) N$_2$ (10 mL/min)
Gradient-application	2 min, A = 50%; B = 50% 5 min, A = 0%; B = 100% 5.5 min, A = 20%; B = 80% 9 min, A = 20%; B = 80% 11 min, A = 50%; B = 50% 15 min, A = 50%; B = 50%	Gas temperature Capillary voltage MRM mode	300 ºC 3500 V 5-FU : 129.0>42.0 (m/z) CP : 261.0>140.0 (m/z) HU : 77.1> 44.0 (m/z)
Flow rate	0.25 mL/min		
Column temperature	40 ºC		
Injection volume	10 µL		

III. RESULT AND DISCUSSION

A. Analytical Method Modification

The simultaneous quantification of 5-FU, CP and HU were performed by applying of mobile phase, gradient and column as shown in TABLE II. The chromatographic conditions were optimized to obtain the better resolution within a shorter analytical time. Two mobile phase systems Milli-Q water + 0.1% formic acid and methanol + 0.1% formic acid were tested result in the best separation of the investigated compounds. For quantitative determination was used electrospray ionization (ESI) for both positive (Cyclophosphamide and Hydroxyurea) and negative (5-Fluorouracil) charges with capillary voltage 3500V. Analyst ions was monitored by using multiple reactions monitoring (MRM) mode. The representative chromatograms were shown in Fig. 3.

Usually, MS/MS can be analyzed both negative and positive charges with short changing time, however, in this case, those 5-FU and HU retention times was overlap, which cannot be analyzed by the usual method. Therefore, we solved this problem by switching detection charges together with multiple injection mode. That mean, during quantification, the first sample injection was detecting positive charge and after CP was detected, the second injection and charge changing were done for negative charge. The procedure mentioned that allows the simultaneous analysis of 5-FU, CP and HU at once.
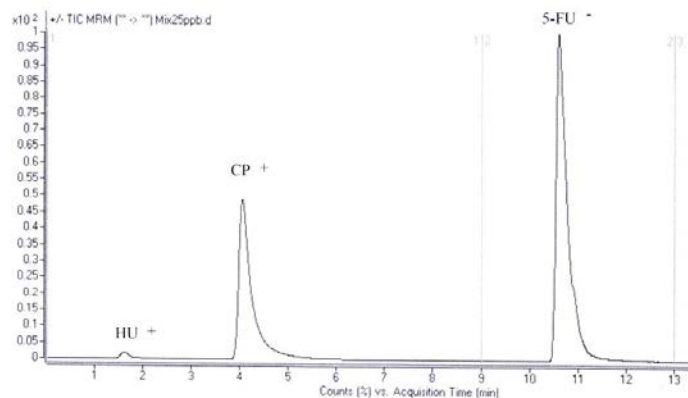

Fig. 3 Representative high performance liquid chromatography (HPLC/MS/MS) chromatograms 5-FU, CP and HU

B. Validation

The linearity was studied in the range from 1 to 50 µg/L of standard 5-FU, CP and HU. Five concentration range were assayed in duplicate. 5-FU, CP and HU standards mixture showed very good linearity. The correlation coefficient (R^2) was always greater than 0.997. Therefore, from results those concentrations with the peak area data measured by this analytical method were correlated and appropriated as shown in Fig 4.
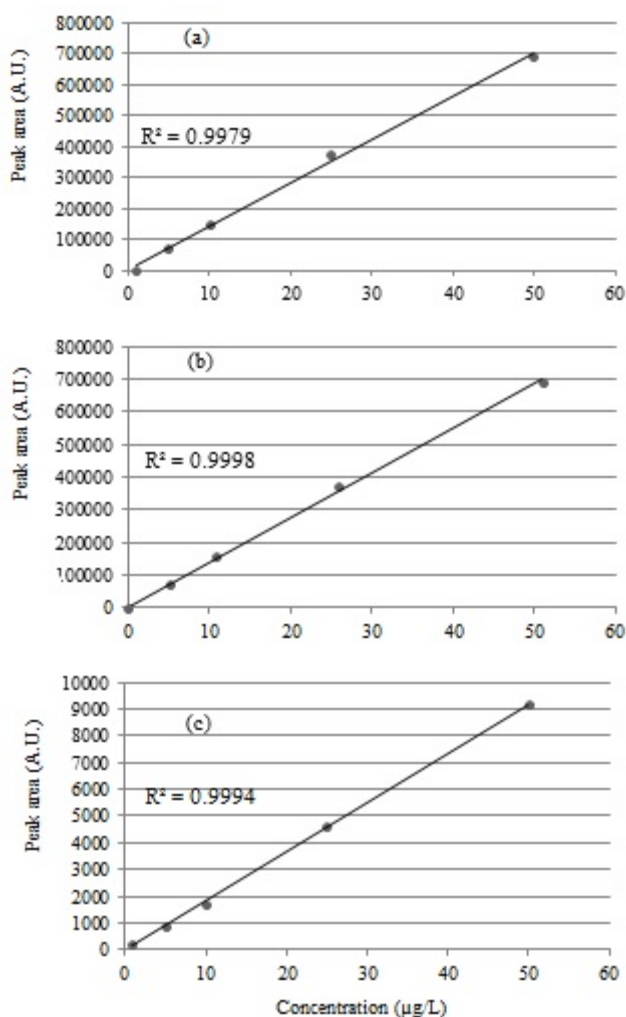
Fig. 4 Linearity of (a) 5-FU, (b) CP and (c) HU

TABLE III
REPRODUCIBILITY

Anticancer drugs (n=5)	Low concentration		High concentration	
	Peak area (Mean)	%RSD	Peak are (Mean)	%RSD
5-FU	78561.55	1.150	221894.17	1.350
CP	69590.67	0.163	177650.02	0.135
HU	65807.73	0.451	126894.67	0.622

TABLE IV
RECOVERY

Anticancer drugs	% Recovery	
	Domestic wastewater	Surface water
5-FU	77	79
CP	108	96
HU	83	104

TABLE V
EVALUATION OF LOD AND LOQ

Anticancer drugs	LOD (µg/L)	LOQ (µg/L)
5-FU	0.013	0.043
CP	0.006	0.020
HU	0.050	0.166

Because the predictive assessment of the situation has limited such as the assumed lower excretion value, wastewater treatment is not available or may disappear in environmental due to the dilution by the natural environment such as rainwater or infiltration. Such phenomenon, the analyzed values may, possibility, found under the estimated. The calculated predicted environmental concentrations of 5-FU, CP and HU in this study were shown similarly to the reported in France and England [21-22]. However, the contamination of 5-FU, CP and HU in surface water might cause the health risk when consumed those water.

TABLE VI
PREDICTED AND MEASUREMENT ENVIRONMENTAL CONCENTRATIONS

Anticancer drugs	Predicted environmental concentrations (µg/L)	Measurement environmental concentrations (µg/L)
5-FU	7.890	0.578
CP	5.750	1.907
HU	3.564	0.788

Reproducibility was calculated by five replications of low and high concentration levels. The relative standard deviation variations of 5FU, CP and HU were 1.350 %, 0.163 % and 0.622 %, respectively. The result shows a good reproducibility and high precision for the quantification of 5-FU, CP and HU (TABLE III) under an agreement between experimental and theoretical values.

Considering on the results of 5-FU, CP and HU were found that the recovery were within the range 77-108%, while the mean recovery at each fortification level and for each sample matrix should be in the range of 70-120% [24]. The recoveries were fall within the acceptable range which indicated that the developed method was reliable and accurate (TABLE IV).

LOD and LOQ of 5-FU, CP and HU with acceptable precision and accuracy, in the present study, were calculated from signal to noise and the data were shown in TABLE V.

C. Predicted environmental concentrations (PECs)

The results from surface water samples were compared to calculated predicted environmental concentrations (PECs) -(1) and the results show that PECs were higher than the actual measured values (Table VI).

IV. CONCLUSION

The study of contamination of 5-FU,CP and HU in water samples can be concluded as follows: Modification and development HPLC-MS/MS method for the simultaneously quantification of 5-FU, CP and HU in water samples were acceptable with high accuracy, precision and specificity for the detection. The linearity was achieved R^2 higher than 0.99 and the calculated LOD for was 5-FU 0.013 µg/L, for CP was 0.006 µg/L and for HU was 0.050 µg/L.

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