DEVELOPMENT AND APPLICATION OF HPLC/MS/MS METHOD FOR DETERMINATION OF HUMAN PHARMACEUTICAL AND SYNTHETIC HORMONES IN STPS EFFLUENTS ALONG LANGAT RIVER AND RECEIVING WATER

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INTRODUCTION

In recent years, the occurrence and fate of Pharmaceutical compounds residues in the environment has become a subject of public interest. When applying Pharmaceutical compounds to humans, many of their constituents are excreted unchanged through urine and feces or as metabolites via municipal sewage system, many Pharmaceutical compounds can frequently be found in effluents of sewage treatment plants (STPs), in rivers and lakes. Balances of the input and the output of drugs and diagnostic agents in STPs reveal that many Pharmaceutical compounds are not removed quantitatively. Therefore STPs act as point sources for surface water contamination.

The reason why Pharmaceutical compounds may be interesting as environmental micropollutants, is that they are developed with the intention of performing a biological effect. They often have the same type of physico-chemical behavior e.g. are lipophilic in order to be able to pass membranes, are persistent in order to avoid the substance to be inactive before having a curing effect as other harmful xenobiotics. Thus, Pharmaceutical compounds have many of the necessary properties to bioaccumulate and provoke effects in the aquatic or terrestrial ecosystems and potential human health risk.

The occurrence and fate of Pharmaceuticals in the aquatic environment has been recognized as one of the emerging issues in environmental chemistry. Recent studies have shown that a multitude of drugs is found in aquatic ecosystems. In some investigations carried out in Austria, Brazil, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, The Netherlands, and the U.S., more than 80 Pharmaceuticals and several drug metabolites, have been detected in the environment. Occurrence of human pharmaceutical pollutants in Malaysian environment has never been studied before except of very few studies on veterinary pharmaceuticals and natural hormones; therefore conducting such study is crucial to have primary information about the pollution status in Malaysia. This study is the first study investigating human pharmaceuticals and synthetic hormones in the Malaysian aquatic environment.

Analysis method used for pharmaceuticals residue in environmental matrices must be highly sensitive and selective for the group of pharmaceuticals of interest. Chemical analysis of pharmaceutical pollutants is very challenging task because unlike other pollutants such as heavy metals or poly aromatic hydrocarbons, which are from the same chemical class, pharmaceutical pollutants usually occurs in groups of different chemical classes. Pharmaceutical of interest varied from country to country as a result of different drugs consumption patterns in each country due to several factors, such as demographic differences, differences in epidemiology of disease,

difference in medical approach, differences in economic conditions or simply because of market reasons. Therefore developing the analytical method that suits the top consumed pharmaceuticals in Malaysia must come first before conducting the monitoring study.

Objectives

1. To develop a method that would be sufficiently sensitive and selective to measure simultaneously a variety of classes of human pharmaceuticals and synthetic hormones in the low ng/L range.
2. To apply the developed method to samples from surface- water and effluent of selected sewage treatment plants along Langat River to determine the concentrations of pharmaceuticals and synthetic hormones
3. Identify the group of pharmaceutical priority pollutants that reflect the prescription and over-the-counter pharmaceuticals most likely found in Malaysian aquatic environment.

Methodology

1. Targeted Pollutants

Targeted pollutants are the top 20 pharmaceuticals consumed by Malaysian based on the ministry of health "medicine use survey 2004" (table 1). Over the counter drugs are not included in this survey, therefore we conducted our survey in the study area namely Kajang, Cheras and Bangi towns to know the most consumed over the counter drugs and hormones (table2).

Table 1: Top 20 drugs by utilization

	Drug	***Therapeutic Class***		***Drug***	***Therapeutic Class***
1	Gibenclamide	Anti- diabetic	**11**	SALBUTAMOL	Anti histamine
2	Atenolol	Beta blocker	**12**	Diclofenac	NSAI
3	Metformin	Anti- diabetic	**13**	Mefenamic Acid	NSAI
4	Mmetoprolol	Beta-blocker	**14**	Loratadine	Antihistamine
5	Nifedipine	Calcium canal blocker	**15**	Furosemide	Loop diuretic
6	Simvastatin	Lipid lowering agent	**16**	INSULINS	Anti- diabetic
7	Amlodipine	Calcium canal blocker	**17**	Cholorothiazide	Diuretic
8	Salbutamol	β2-Sympathomimetic	**18**	Lovastatain	Lipid-lowering drug
9	Chlorpheniramine	antihistamine	**19**	Amoxicillin	Antibiotic
10	Gliclazide	Anti- diabetic	**20**	Perindopril	Antihypertensive

Table 2: Top over the counter drugs sold in pharmacies around the study area

	Drug	***Therapeutic Class***
1	Pacacetamol	analgesic
2	Acetylsalicylic acid	analgesic
3	Desogestrel	hormonal contraceptive
4	Levonorgestrel	hormonal contraceptive
5	Drospirenone	hormonal contraceptive
6	Ethinylestradiol	hormonal contraceptive
7	Cyproterone	hormonal contraceptive
8	Norethisterone	hormonal contraceptive

2. Experimental part

2.1. Method development, optimization and validation

Analytical procedure is summarized in figure 1.

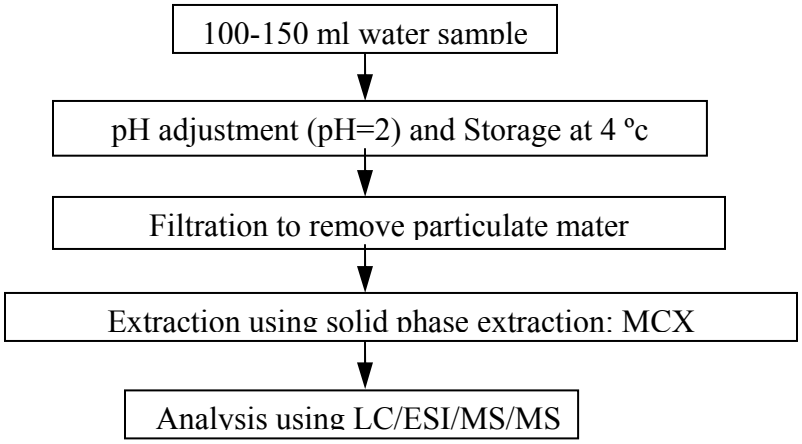

Figure 1: summary of proposed analytical technique for analyzing pharmaceutical and hormones in water samples.

Method development and optimization will include the extraction step and the analysis using high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) technique. The last tow steps in the proposed analytical technique (figure 1) must be first developed and validated as following:

1. MS/MS Parameters Optimization :
   a. Determination of the best ESI ionization mode (- or +)
   b. Optimization of compound-dependent parameters (precursor ion, product ions, collision energies CE and cone voltage).
   c. Optimization of MRM for quantitation and confirmation.
2. Development and optimization of the HPLC separation method (solvents, buffer, additives and the gradient profile for the elution) in both negative and positive ion modes.

Ultimately developed method was validated using the US Environmental Protection Agency (USEPA) protocol whereby the instrumental and method parameters will be tested for accuracy and precision.

2. Method application

2.1. Study Area

Study conducted on selected STPs in Langat basin and on receiving surface water of the Langat River.

2.2 Sample Collection

150 ml of surface water samples collected from five locations along the river and 100 ml STPs effluents samples collected from five selected STP in Langat basin. Samples kept cool (4 ˚C) until analysis. Analysis conducted following the proposed protocol summarized in figure 1.

Result and discussion:

1. Method development

1.1 MS/MS optimization:
Upon receiving of the standard reference material (purity >98%) of the targeted compounds (table 1 and 2), MS/MS parameters were successfully optimized for all of the analytes and subsequently divided to two groups based on their ionization response (positive ions and negative ions). Mass spectrometry analysis was performed using a Micromass Quattro Ultima Pt tandem triple quadrupole mass spectrometer (Waters, MA, USA), equipped with Electrosperay Ionization (ESI).

2.1 HPLC method development

1.1.1. Ions mixture detected in –ve mode:

Waters Alliance HPLC System 2695 Separations Module from Waters (Milford, MA, USA) was used to develop the liquid chromatography method. Separation method was successfully developed for the ions detected in the –ve ESI mode using 100 x 2.1 mm, 3.5 um Zorbax Extened-C18 column (Agilant, USA).The liquid phase used is a binary gradient consisting of (A) MilliQ water with 5mM ammonium and 0.1% of TBA and (B) 66%/34% of ACN/ MeOH Separation for 8 compounds in only 10 minutes is shown in separate channels in figure 2.

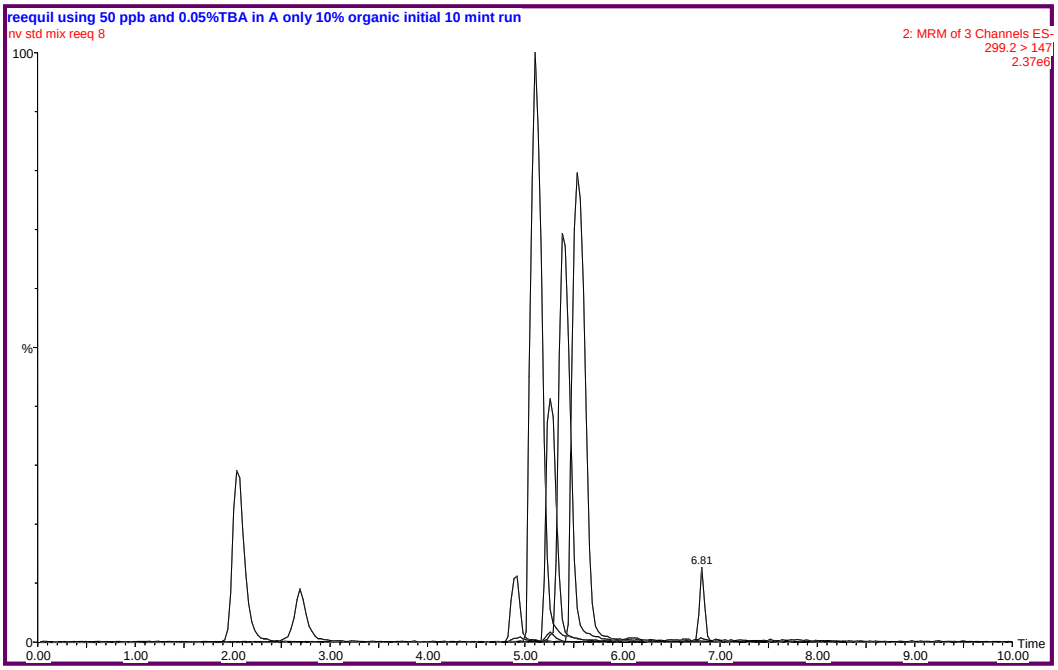

Figure 2: TIC for 8 compounds detected in –ve ion mode

1.1.2 Ions mixture detected in +ve mode:

Separation for 15 compounds detected in the +ve ESI mode was done using 100 x 2.1 mm, 1.8 um Zorbax SB-C18 column(Agilant, USA) with only 22 mints time. The liquid phase used is a binary gradient consisting of (A) MilliQ water with 5mM ammonium and 0.1% of HFBA and (B)

66%/34% of ACN/ MeOH. Separation for 15 compounds in only 20 minutes is shown in separate channels in figure 3.

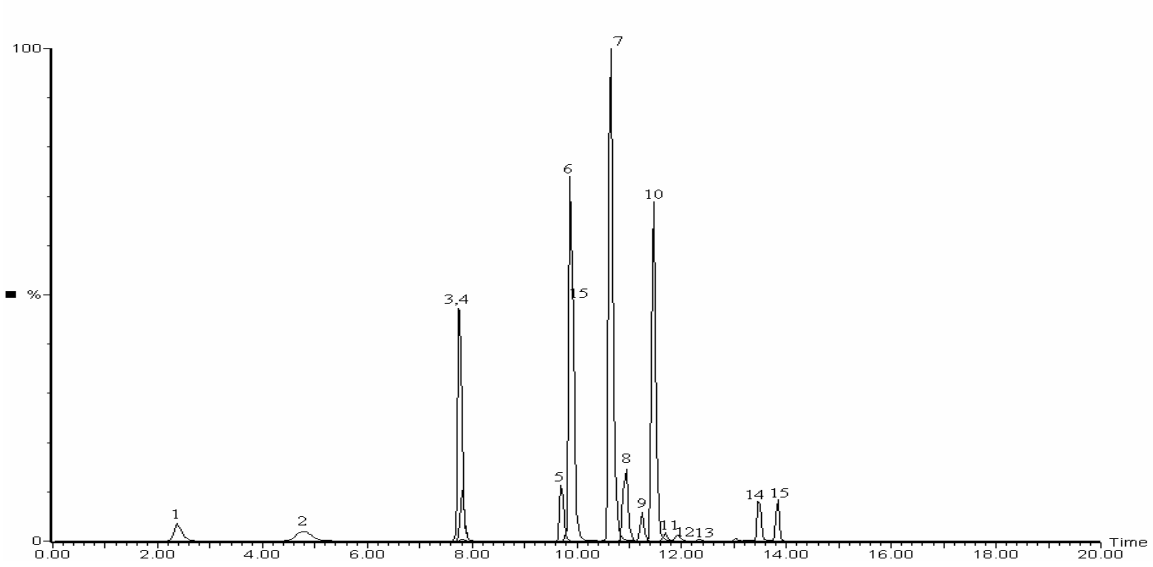

Figure 3: TIC for 15 compounds detected in +ve ion mode.

2. Method validation:

2.1. Linearity

HPLC/ESI/MS/MS method was found to be linear over wide range of concentration (up to 5000 ppb) with r^2 exceeding 0.99 for most of the compounds.

2.2. Sensitivity (LOD &LOQ):

IDL is as low as pg on column level for most of the compounds and limit of quantification is as low as 1 ng/L for most of the compounds.

2.3 Recovery %

Recovery of the whole method has been tested against the ultra pure water, control water and real samples at 100µg/L level. Recovery was found to be between 80-110 % for ultra pure water and control water and 40-120 % for real samples.

The overall results of the method validation show that the developed method is accurate and sensitive to be used for trace analysis for the real environmental samples.

3. Occurrence of pharmaceuticals in River and STP effluents samples collected form Langat basin.

3.1 River samples

All of the samples collected from the surface water found to be impacted by most of the pollutants under study. The occurrence and concentration of some of these pollutants is summarized in figure 4.

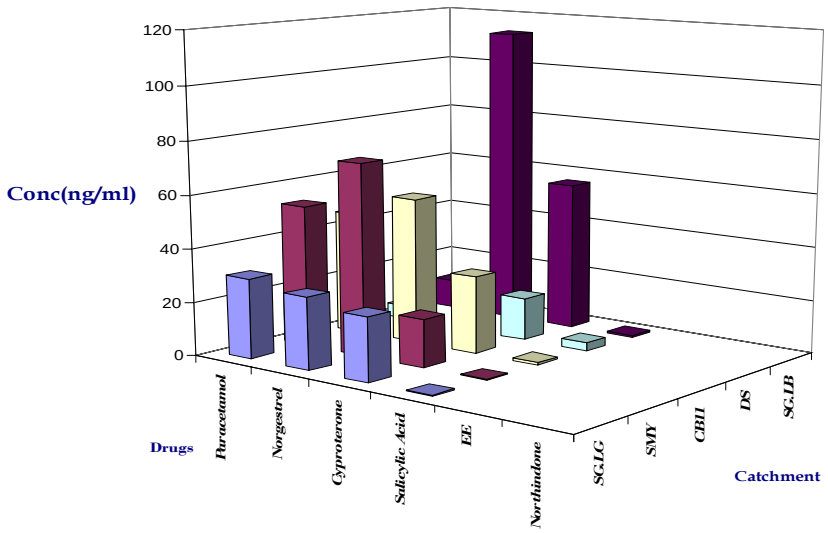

Figure 4: levels of some pharmaceuticals in samples collected from Langat River

3.1 STPs effluent samples

All of the samples collected from STPs effluents found to be impacted by most of the pollutants under study. The occurrence and concentration of some of these pollutants is summarized in figure 5.

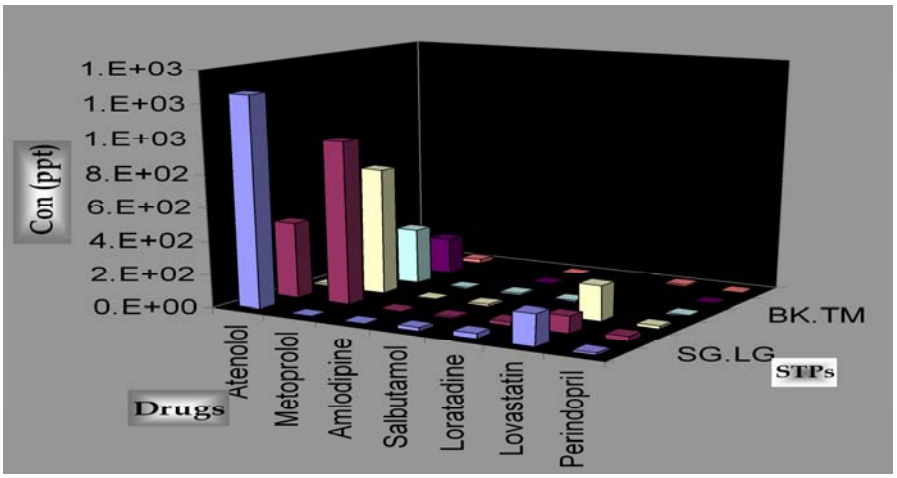

Figure 5: levels of some pharmaceuticals in samples collected from STP

Significance of finding

It should be recalled that pharmaceuticals and synthetic hormones lately have been acknowledge to constitute a major health risk for human and terrestrial and aquatic ecosystems. Data about the pharmaceuticals pollution in Malaysian aquatic environment is missing; therefore developing a method that can simultaneously detect and precisely quantify the concentration of 23 different compounds in the aquatic matrix is of great importance to reveal the status of pollution.

The developed LC/ESI/MS/MS method was found to be sensitive, fast and efficient for analysis of 23 human pharmaceutical and synthetic hormones in only 30 minutes total analysis time. Applying this method on environmental samples revealed that the level of pharmaceuticals and hormones concentration in Malaysian aquatic environment can be found at µg/L.