Direct Analysis of Six Pharmaceuticals using Online Solid Phase Extraction Liquid Chromatography

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Abstract: In this study, three parameters (sample volume, cleanup and elution step) in large volume injection online Solid Phase Extraction Liquid Chromatography with diode array detection (SPE-LC) were optimized for the determination of six pharmaceuticals in wastewater samples. Preconcentration and separation were carried out using 2 columns, Dionex Ion Pac AG14A and Acclaim Polar Advantage II. Response Surface Methodology (RSM) was employed for the optimization of cleanup step (mobile phase composition and valve switching time). The optimum conditions obtained were as follows: Sample volume of 10.0 mL and cleanup with acetonitrile and ultrapure water (5:95) for 1.0 min. The method showed good recovery (82.91-107.7%), precision (0.35-8.26%) and linearity ($R^2>0.99$). The limit of detection was between 3.5 and 11.7 µg L$^{-1}$. The proposed method is rapid, simple and sensitive for trace analysis of pharmaceuticals in water samples.

Keywords: Online SPE-LC, Optimization, Water Sample, Pharmaceuticals

Introduction

In recent years, the occurrence of pharmaceuticals in the aquatic environment has raised concerns (Cardoso et al., 2014; Devier et al., 2013; Lindberg et al., 2014; Repice et al., 2013; Schaidter et al., 2014; Vulliet and Croen-Olive, 2011). Pharmaceuticals and related compounds enter the water compartment mainly through municipal wastewater, hospital waste and pharmaceutical industries effluent (Golovko et al., 2014; Verlicchi et al., 2010). They may pass through the conventional water treatment plant and eventually, presence in drinking water (Rivera-Utrilla et al., 2013).

As a result of their occurrence, analytical techniques for trace analysis of wide range pharmaceuticals in water samples, such as wastewater, has been extensively investigated for better efficiency and sensitivity (Buchberger, 2011; Ding et al., 2013; Pailler et al., 2009; Pavlović et al., 2007; Robles-Molina et al., 2014; Yu and Wu, 2011). The concentration of detected pharmaceuticals in wastewater dependson type of treatment used. Based on recent studies, Baker and Kaszprzyk-Hordern (2013) reported high and low concentrations of caffeine in influent wastewater sample (23.77 µg L$^{-1}$) and in effluent (1.74 µg L$^{-1}$).

Solid phase extraction is the most used method for extraction of pharmaceuticals in water samples. However, an alternative to conventional SPE, online Solid Phase Extraction Liquid Chromatography (SPE-LC) has been getting more attention since it shortens the extraction and analysis time by its automated technique and reduces the sample volume. Switching valve time is used to perform the extraction and separation in online SPE-LC. The on column preconcentration of high sample volume injection (up to 10 mL) in online SPE-LC method showed better sensitivity compared to small volume sample injection (Lindberg et al., 2014; Liu et al., 2013). Hence, optimizing Large Volume Sample Injection (LVSI) method, sample loading volume and cleanup steps need to be considered in developing online solid phase extraction method (Fernández-Ramos et al., 2014). However, sample loading volume should not exceed maximum adsorption capacity of extraction column and cleanup step is required to eliminate interference matrix components. During the cleanup step, the choice of mobile phases and time of valve switching are important factors that influence the extraction recovery (Fernández-Ramos et al., 2014). Response Surface Methodology (RSM) based on a Central Composite Design (CCD) evaluates the effects of variables using minimum number of experiments. This approach has been applied in the optimization of the extraction method and chromatographic separation (Xu et al., 2014; Bezerra et al., 2008).

Our work attempted to develop LVSI online SPE-LC to detect common pharmaceuticals (acetaminophen,
caffeine, carbamazepine, naproxen, diclofenac and ibuprofen). RSM was applied in determining the optimum composition of mobile phase and valve switching time. The optimized method was validated and applied to wastewater, surface water and upstream water samples.

**Experimental**

**Chemicals and Materials**

Standard pharmaceuticals (acetaminophen (ACT), caffeine (CAF), carbamazepine (CBZ), naproxen (NAP), ibuprofen (IBU) and diclofenac (DICLO)) and methanesulfonic acid (MSA) were purchased from Sigma-Aldrich (purity assay in range of 98-101%) (St. Louis, USA). Acetonitrile (ACN) (HPLC grade) was purchased from Merck (Darmstadt, Germany). Hydrochloric acid (HCl) (37%) was from Merck, Darmstadt, Germany. Ultrapure water was produced with aBarnstead Nanopure system (Thermo Scientific, USA).

**Online Solid Phase Extraction Liquid Chromatography (SPE-LC) System**

Solid phase extraction and chromatographic separation was performed using an automated online SPE-LC Dionex Ultimate 3000 (Sunnyvale, CA, USA) system. The system consists of dual gradient pumps, left and right, a solvent rack with an integrated vacuum degasser, a large column compartment, two columns (online SPE column and analytical column) and a diode array detector. The online SPE column was an IonPac AG14A RFIC Guard (4.0×50 mm) (Thermo Scientific USA) with the left pump using the conditioning solution (10 mM MSA, 5:95 (v/v) and kept for 1.0 min to remove any possible impurities retained together with analytes.

**Elution and LC Separation**

After cleanup, the valve was switched to connect the SPE column with the analytical column to elute analytes from SPE column into analytical column (Acclaim Polar Advantage II, 5 µm, 120 Å, 4.6×150 mm, Thermo Scientific USA), where the separation occurred simultaneously. The elution started with LC mobile phase at ACN: 10 mM MSA (30:70) followed by a linear gradient to ACN: 10 mM MSA (70:30). Then, the valve was switched to disconnect the online SPE column and analytical column. The separation of analytes continues using LC mobile phase from ACN: 10 mM MSA (70:30) increases to 100% ACN in 3 min and was maintained for 3 min. Meanwhile, the online SPE column was equilibrated with initial conditioning solution. The syringe, injection valve and sample loop were programmed to be washed to eliminate carry over.

**Detection**

Pharmaceuticals were detected simultaneously at various wavelengths. The Diode Array Detector (DAD) was set at 250 nm (acetaminophen), 280 nm (caffeine, carbamazepine, ibuprofen) and 220 nm (diclofenac, naproxen). Identification of pharmaceuticals was based on retention time and Ultraviolet (UV) spectrum of each pharmaceutical.

**Preparation of Calibration Standards and Spiked Samples**

The individual stock solution (1 mg mL⁻¹) of pharmaceuticals was prepared in methanol and stored in amber glass bottle. A series of working standard solutions (0.1-50.0 µg L⁻¹) were prepared in ultrapure water by dilution prior to analysis to prevent decomposition of analytes. The pH of the ultrapure water was adjusted to pH 2 with HCl (3 M) (optimum condition for the analysis). The wastewater (adjusted to pH 2) was spiked with the standard solutions of the pharmaceuticals to obtain two concentrations (5.0 and 50.0 µg L⁻¹) for accuracy and precision studies. The chromatogram of standard mixture is shown in Fig. 1.

**Sample Collection and Preparation**

Wastewater samples were collected from 15 wastewater treatment plants in Malaysia. Influent wastewater samples were collected using the grab sampling technique and transferred into a 1.0 L high-density polyethylene bottle and were immediately transported to the laboratory. Water samples were acidified to pH 2 using HCl (3M). The samples were vacuum filtered using Whatman 0.45 µm Glass Fiber (GF/A) (Whatman International Ltd Maidstone, England) to remove suspended solid matter. The filtered water samples were kept in the dark and stored at 4°C prior to analysis.
Table 1. Results from Central Composite Design (CCD)

| Compounds        | Source | p-value | Lack of fit | Significant model terms |
|------------------|--------|---------|-------------|-------------------------|
| Caffeine         | Linear | 0.0117  | 0.4324      | A, B                    |
| Acetaminophen    | Linear | 0.0154  | 0.3244      | B                       |
| Carbamazepine    | Mean   | -       | -           | -                       |
| Naproxen         | Quadratic | 0.0926 | 0.0583      | -                       |
| Diclofenac       | Quadratic | 0.0792 | 0.9382      | AB                      |
| Ibuprofen        | Quadratic | 0.3286 | 0.0003      | -                       |

*A: Acetonitrile composition; B: Valve switching time

**Method Validation**

Method validation was achieved using external calibration curve. Calibration solution of the analytes of 0.1, 0.5, 1.0, 5.0, 10.0, 50.0 µg L\(^{-1}\) were prepared by dilution of working standard mixture of 100 µg L\(^{-1}\). Methanesulfonic acid, 10 mM in ultrapure water was added to all samples. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated using linear regression method. Intra-day and inter-day precisions were determined for a concentration of 5 µg L\(^{-1}\) of standard mixture in ultrapure water and influent wastewater. Intra-day precision was calculated as RSD in percentage of the peak area of analytes (n = 5). The inter-day precision was determined by analyzing the spiked water over five consecutive days. The accuracy was determined by extraction recoveries of pharmaceuticals in wastewater spiked with standard mixtures (5 and 50 µg L\(^{-1}\)). System suitability studies for the method were carried out to determine the resolution, theoretical plates, repeatability of retention time and tailing factor.

**Results and Discussion**

**Online SPE Optimization**

**Sample Volume**

The amount of sample pre-concentrated on SPE column affects the sensitivity of the method (Ferreira et al., 2012). The effect of sample loading volume on peak area of analyte pharmaceuticals was studied by evaluating 1.0, 5.0 and 10.0 mL of spiked ultrapure water. The peak area of analytes increases with increasing sample volume, thus increasing the sensitivity of the method (Fig. 2). Therefore, a sample volume of 10 mL was selected for subsequent optimization study.

**Composition of Mobile Phase for Cleanup**

The objective of cleanup step is to remove interferences from the extraction column. Two significant variables for cleanup step, mobile phase composition and valve switching time (Liu et al., 2013) were optimized using central composite design. The
mobile phase composition of acetonitrile and ultrapure water were evaluated in the range of 5-95% and the valve switching time was in the range of 1.0-3.0 min. The flow rate of system was maintained at 1 mL min$^{-1}$ to prevent flow inconsistency. The significance of the model was assessed by F-ratio at probability (p) of 0.05, whereby values less than 0.05 indicate the significance of the model. A non-significant for lack of fit indicated that the model was valid to the spatial influences of variables on the responses (Liu et al., 2013). From the results of six responses (Table 1), caffeine and acetaminophen showed a significant linear model (p = 0.0117) with not significant lack of fit (p = 0.4324). The results indicated the adequacy of the applied linear model. The predicted values of caffeine and acetaminophen were calculated using the equation according to the desirability function and compared with experimental value to obtained optimized condition of cleanup step. With desirability function of 0.759, the composition of acetonitrile to ultrapure water (5:95) and switching valve time of 1.0 min were selected for cleanup step.

The optimized cleanup step was tested using spiked wastewater sample. Figure 3 clearly shows the removal of matrix interferences with cleanup step. The method with cleanup step increased analysis time by 1 min compared to the method without cleanup step. The proposed online SPE-LC method has successfully separate and detect caffeine in the wastewater sample.

**Elution Solvent and Valve Switching Time**

In optimizing elution solvent and valve switching time, various isocratic elution compositions of acetonitrile and 10 mM MSA were studied. Figure 4a-c show that acetaminophen and caffeine were eluted with 20-40% of ACN, while carbamazepine and naproxen were eluted within 30-70% and 60-70% of ACN, respectively. Diclofenac and ibuprofen were eluted from the column within 60-70% of ACN. A decrease in composition of 10 mM MSA with increase of ACN improved the separation of the pharmaceuticals compounds. Hence, a gradient elution approach was performed (Fig. 4d). Optimized gradient elution with initial composition 30:70 (ACN:MSA) to final composition of 70:30 (10% ACN min$^{-1}$) with switching time of 4 min was able to elute all selected pharmaceuticals (Fig. 4d).

**Method Validation**

An external calibration curve was generated using five concentrations of standard mixtures in the range of 0.1 to 50 0 µg L$^{-1}$ with three replications. Linear curves for each pharmaceutical were obtained between peak areas with a good correlation coefficient ($R^2 = 0.992-0.999$) (Table 2). The sensitivity of method expressed as Limit of Detection (LOD) and Limit of Quantification (LOQ) values were calculated based on linear regression method ranges from 2.5-11.7 and 7.5-35.4 µg L$^{-1}$, respectively. The LOD and LOQ for this method were lower than those reported whereby the LOD and LOQ ranges from 6-74 and 60-200 µg L$^{-1}$, respectively (Chandra and Dutt Sharma, 2013; Stafiej et al., 2007). The LOD and LOQ values are also comparable to those reported in the literature (Aguilar-Arteaga et al., 2010). The intra-day and inter-day repeatability were conducted to evaluate the precision of this method. The results were expressed as % RSD based on the peak area. The intra-day was calculated by performing the analysis of spiked ultrapure water sample and inter-day repeatability study was performed within five consecutive days. The results for intra-day (0.67-7.87%) and the inter-day (3.47-9.86%) (Table 3) showed good precision due to less labor and the fully automatic procedure in the online SPE-LC procedure (Negreira et al., 2013).
The accuracy was determined by extraction recoveries of pharmaceuticals in wastewater spiked at concentration of 5 and 50 µg L\(^{-1}\). Good recoveries of 82.91-107.7% with % RSD of 0.35-8.26% were obtained for all analytes. The result concurred with the study by Al-Odaini et al. (2013). Results for system suitability studies (Table 4) demonstrated the suitability of the developed method for the analysis of the six pharmaceuticals.

**Analysis of Water Samples**

The developed method was successfully applied to analyze the six pharmaceuticals in wastewater samples from several influent wastewater treatment plants of Selangor, Malaysia. Concentrations of pharmaceuticals in wastewater are presented in Table 5.

The most frequently detected compounds in influents were acetaminophen, caffeine and diclofenac. Levels detected were in the range of 0.58 to 49.9 µg L\(^{-1}\). High concentration of acetaminophen can be explained by its widespread use as an effective painkiller in Malaysia. Acetaminophen (commercially known as paracetamol) and aspirin are the most popular analgesics, available over the counter medication and no prescription is needed upon purchased (Al-Odaini et al., 2013; Papageorgiou et al., 2016). Caffeine was present in all influent samples as it is used in beverages and food as a stimulant. Previous studies reported higher concentration for acetaminophen (134 µg L\(^{-1}\)) (Gracia-Lor et al., 2012) and caffeine (96.6 µg L\(^{-1}\)) (Kosma et al., 2014) in influents. The concentration of diclofenac in influent samples in this study was comparable or higher than maximum concentration observed by Kosma et al. (2014) (5.16 µg L\(^{-1}\)) and Papageorgiou et al. (2016) (4.87µg L\(^{-1}\)). Carbamazepine, naproxen and ibuprofen were not detected at concentration maybe below the LOD (3.5-5.7 µg L\(^{-1}\)) of the developed method. Very low concentration of carbamazepine, naproxen and ibuprofen were reported by other studies ranged from 0.07-3.0 µg L\(^{-1}\) (Behera et al., 2011; Blair et al., 2013).
Fig. 4. Chromatograms of isocratic elution, (a) 30% ACN: 70% MSA; (b) 60% ACN: 40% MSA; (c) 70% ACN: 30% MSA and gradient elution (d) 30 to 70% of ACN: 70 to 30% MSA. 1: Caffeine, 2: Acetaminophen, 3: Carbamazepine, 4: Naproxen, 5: Diclofenac, 6: Ibuprofen

Table 2. Coefficients of determination, linear range, LOD and LOQ

| Pharmaceutical | Linear range (µg L\(^{-1}\)) | Coefficients of determination (R\(^2\)) | LOD (µg L\(^{-1}\)) | LOQ (µg L\(^{-1}\)) |
|----------------|-----------------------------|----------------------------------------|---------------------|---------------------|
| Acetaminophen  | 0.1-50                      | 0.995                                  | 11.7                | 35.4                |
| Caffeine       | 0.1-50                      | 0.999                                  | 2.5                 | 7.5                 |
| Carbamazepine  | 0.1-50                      | 0.996                                  | 5.7                 | 17.4                |
| Diclofenac     | 0.5-50                      | 0.997                                  | 4.2                 | 12.6                |
| Ibuprofen      | 0.1-50                      | 0.998                                  | 3.5                 | 10.5                |
| Naproxen       | 0.1-50                      | 0.999                                  | 5.5                 | 16.7                |

Table 3. Accuracy and reproducibility (%RSD) in spiked wastewater

| Pharmaceutical | Recovery (%) | Intra-day\(b\) | Inter-day\(b\) |
|----------------|--------------|----------------|----------------|
| Acetaminophen  | 107.7 (0.8)  | 4.54           | 9.86           |
| Caffeine       | 106.8 (0.72) | 5.45           | 3.59           |
| Carbamazepine  | 82.91 (4.41) | 7.87           | 6.28           |
| Naproxen       | 98.45 (1.42) | 1.37           | 3.70           |
| Diclofenac     | 98.56 (6.13) | 4.92           | 3.47           |
| Ibuprofen      | 102.4 (6.05) | 0.67           | 5.76           |

\(^a\) RSD (%) value is in bracket
\(^b\) \(n = 5\)
Conclusion

The online SPE-LC method was able to provide rapid analysis for pharmaceuticals in wastewater with good reproducibility and accuracy. As the sensitivity obtained is within µg L$^{-1}$ level, the method is therefore suitable for trace analysis. Large volume sample injection improves sensitivity of the method while online sample preconcentration reduce pretreatment and analysis time. Column switching system allows automated sample preparation and separation without manual labor thus reducing human error. The IonPacAG14A column could be used repeatedly under proposed experimental conditions for over 300 injections of 10 mL of environmental water samples.

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Author’s Contributions

Siti Norbayu Mohd Subari: Performed the method development experiments, analyzed the data and wrote the manuscript.

Norashikin Saim: Guided the experimental work and revised the writing of manuscript.

Rozita Osman: Guided the experimental work and revised the writing of manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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