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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; Schaider *et al*., 2014; Vulliet and Cren-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 Kasprzyk-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 cleanupsteps 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 volume loop (10.2 mL) autosampler, a thermostated 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) and analytical separation was an Acclaim Polar Advantage II (5 µm, 120 Å, 4.6×150 mm) (Thermo Scientific USA) as the analytical column. The system is equipped with a programmable 6ports/ 2 position switching valve for several modes (loading, washing, elution and separation). Data were processed by the Chromeleon Software v.6.8 (Dionex).

The method used four major steps: Sample loading, cleanup, elution and LC separation. Both pumps ran simultaneously. The flow rate was set at 1 mL min^{-1} throughout the analysis andtemperature was set at a 40°C.

Sample Loading and Cleanup

About 12 mL water sample was drawn into high volume loop (10 mL) by a syringe from a 25 mL vial. The sample loop was overfilled with water sample to eliminate wash solution presence in sample loop and to ensure only water sample inside the sample loop. In the sample loading step, 10 mL water sample was loaded onto online SPE column (IonPac AG14A Guard (4.0×50 mm, Thermo Scientific USA) with the left pump using the conditioning solution (10 mM MSA in H_2O) at flow rate of 1 mL min^{-1}. After sample loading, the valve was switched to cleanup step using the cleanup mobile phase (ACN: 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 startedwith 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 variouswavelengths. 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 andstored 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 pH2 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.

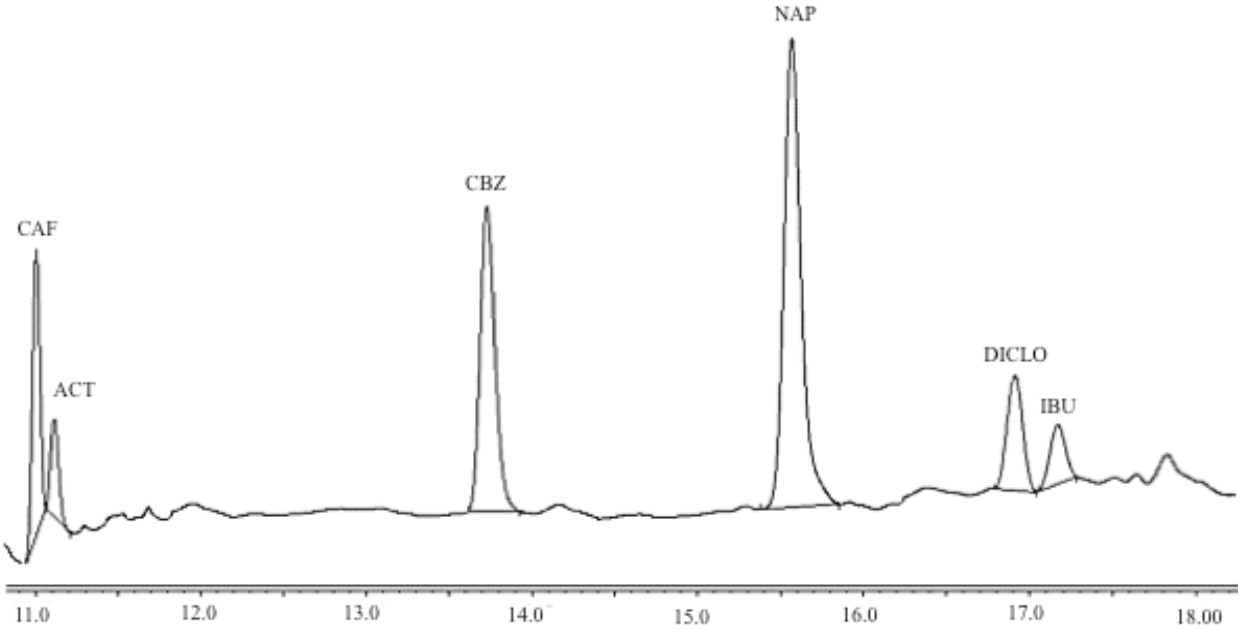

Fig. 1. Chromatogram of standard pharmaceuticals (5 μg L^{-1})

Table 1. Results from Central Composite Design (CCD)

	Model			
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 werecarried 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 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. Anon-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).

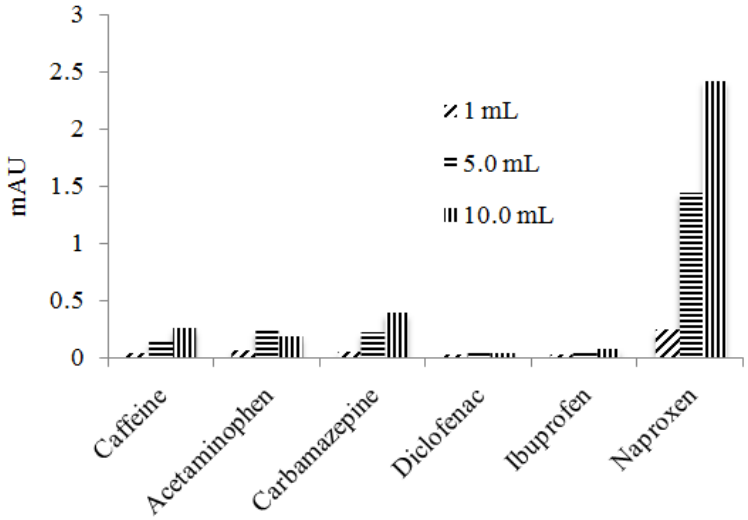

Fig. 2. The effect of different sample volume on the amount of pharmaceuticals

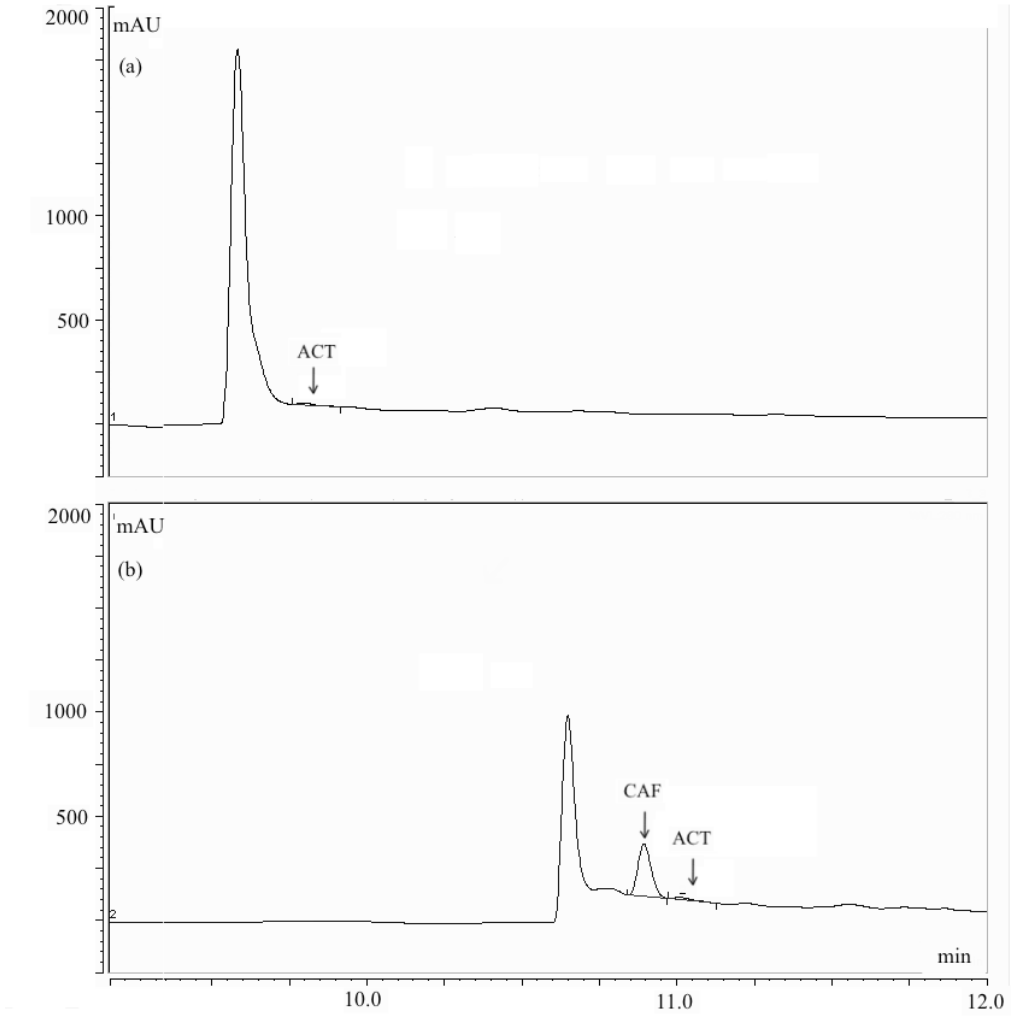

Fig. 3. Chromatogram of spiked wastewater sample without cleanup step (a) and with cleanup step (b) Sample volume: 5 mL

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).

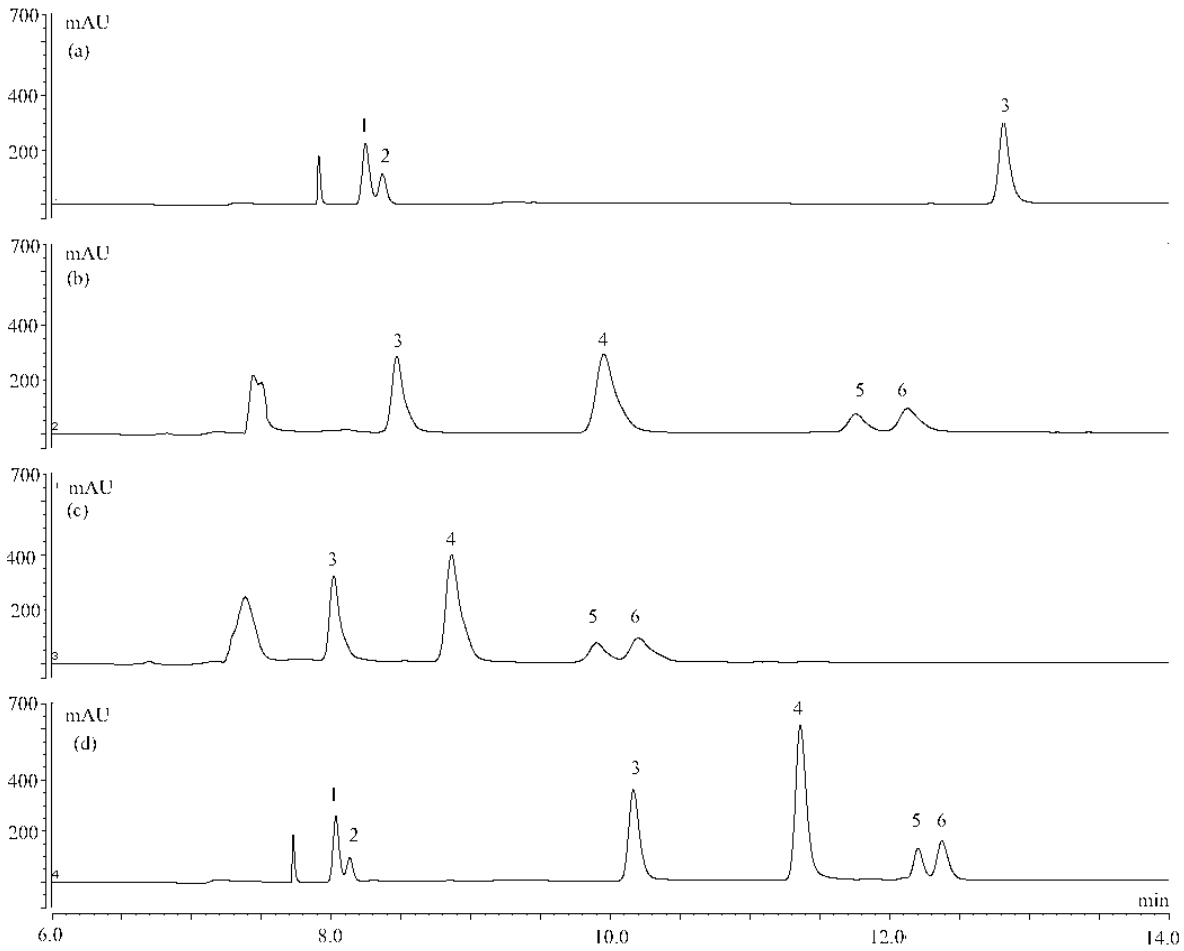

Fig. 4. Chromatograms of isocratic elution, (a) 30% ACN: 70% MSA; (b) 60% ACN: 40% MSA; (c) 70% ACN: 30% MSA and gradient elution (d) 30 to 70% of ACN: 70 to 30% MSA. 1: Caffeine, 2: Acetaminophen, 3: Carbamazepine, 4: Naproxen, 5: Diclofenac, 6: Ibuprofen

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

	Recovery (%)			
Pharmaceutical	5 (μg L^{-1})	50 (μg L^{-1})	Intra-day[b] 50 (μg L^{-1})	Inter-day[b] 50 (μg L^{-1})
Acetaminophen	107.7 (0.8)	95.26 (1.38)	4.54	9.86
Caffeine	106.8 (0.72)	93.74 (3.99)	5.45	3.59
Carbamazepine	82.91 (4.41)	96.62 (1.05)	7.87	6.28
Naproxen	98.45 (1.42)	92.20 (1.65)	1.37	3.70
Diclofenac	98.56 (6.13)	89.75 (8.26)	4.92	3.47
Ibuprofen	102.4 (6.05)	83.67 (0.35)	0.67	5.76

*RSD (%) value is in bracket
[b]n = 5

Table 4. System suitability studies

Pharmaceutical	Resolution	Theoretical plates	Tailing factor
Acetaminophen	16.4	175266	0.75
Caffeine	6.14	165311	0.90
Carbamazepine	8.32	71053	1.20
Diclofenac	1.08	93323	0.86
Ibuprofen	-	54558	0.86
Naproxen	5.793	65263	1.00

Table 5. Concentrations (μg L^{-1}) of pharmaceuticals in samples

	Compounds					
Sampling site	Caffeine	Acetaminophen	Carbamazepine	Naproxen	Ibuprofen	Diclofenac
S1	14.21	10.97	n.d	n.d	n.d	2.36
S2	7.78	41.65	n.d	n.d	n.d	12.82
S3	0.58	39.01	n.d	n.d	n.d	n.d
S4	8.13	11.05	n.d	n.d	n.d	n.d
S5	7.78	6.19	n.d	n.d	n.d	8.59
S6	7.70	44.66	n.d	n.d	n.d	n.d
S7	4.89	12.05	n.d	n.d	n.d	n.d
S8	9.51	19.94	n.d	n.d	n.d	24.30
S9	5.54	19.94	n.d	n.d	n.d	1.66
S10	11.87	8.83	n.d	n.d	n.d	n.d
S11	7.34	49.97	n.d	n.d	n.d	2.59
S12	8.51	9.14	n.d	n.d	n.d	2.06
S13	6.49	9.09	n.d	n.d	n.d	13.62
S14	15.69	12.53	n.d	n.d	n.d	n.d
S15	8.30	9.45	n.d	n.d	n.d	1.29

*n.d: Non detected (below the detection limit)

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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