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Simultaneous determination of paracetamol and diclofenac in wastewater by high-performance liquid chromatography method

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Abstract. In this study, a simple, selective, and accurate analysis method was developed for the simultaneous determination of anti-inflammatory drugs (paracetamol and diclofenac) existing inlet and outlet of the wastewater treatment system. This method used solid-phase extraction (SPE) and reversed-phase high-performance liquid chromatography with a photodiode array detector (HPLC-PDA). Separation was performed using an HSR C18 column (250 mm × 4.6 mm; 5 µm) with a mobile phase consisting of acetonitrile: sodium dihydrogen orthophosphate in the ratio 60:40 v/v. The flow rate was 1 ml/min, and PDA was detected at 243 nm with paracetamol and 276 nm with diclofenac. The duration of paracetamol and diclofenac was 2.39 and 4.39 min, respectively. The developed method was validated in terms of accuracy, precision, and linearity for two drugs found in the range of 0.01 - 1 µg/ml, and 0.02 - 1 µg/ml for paracetamol, and diclofenac, respectively. The limits of detection for paracetamol and diclofenac were 0.003 µg/ml and 0.006 µg/ml, respectively, whereas the limits of quantification were 0.01 µg /ml and 0.02 µg/ml, respectively. The recoveries for paracetamol and diclofenac were greater than 95 %. The described method also has been applied to determine paracetamol and diclofenac simultaneously in the wastewater of the treatment system.

Keywords: HPLC, paracetamol, diclofenac, wastewater.

Classification numbers: 3.2.1, 3.3.1, 3.4.2.

1. INTRODUCTION

The growing usage of pharmaceutical compounds in humans and animals has posted a new environmental issue. Several studies have shown that nonsteroidal anti-inflammatory drugs, analgesics, and antipyretics are among the pharmaceutical residues which appear in the environment and cause potential harm to humans and ecosystems. They occur in concentrations ranging from ng/l to mg/l in the different aquatic environments [1 - 4]. Pharmaceutical residues in the environment affect the fertility and survival of organisms and ecosystems, leading to

indirect effects on the human body [5]. One of the most used drugs today is paracetamol and diclofenac.

Diclofenac sodium or sodium [O-(2,6-dichlorophenyl)-amino-phenyl] acetate (DIC) is a nonsteroidal anti-inflammatory analgesic with potent cyclooxygenase inhibitory activity. This medication is commonly used to control pain and treat rheumatic diseases. Diclofenac is well absorbed after oral administration and is metabolized by the liver [6].

Paracetamol or 4-hydroxy acetanilide (PAR) is a para-aminophenol derivative with analgesic and antipyretic properties but no anti-inflammatory properties. The reaction mechanism of paracetamol is due to its inhibition of the cyclooxygenase enzyme and prostaglandin synthesis in the central nervous system and its direct activity on the hypothalamic center for body temperature regulation [6].

Pharmaceutical quantitative method research is critical in monitoring and correctly quantifying pharmaceutical residues in the environment. Several methods for quantifying paracetamol and diclofenac have been employed, including electrochemical methods [7], titration methods [8]; spectroscopic methods [9, 10], fluorescence methods [11, 12] and chromatographic methods [13, 14].

Because of its selectivity, high sensitivity, rapid analysis time, simplicity of operation, and capability of simultaneous analysis, high-performance liquid chromatography is frequently employed in labs for drug pharmacokinetic study. However, in Viet Nam, there are no studies to simultaneously identify PAR and DIC in water samples to assess pharmaceutical residues in the environment. Therefore, a method for the simultaneous determination of PAR and DIC in wastewater by high-performance liquid chromatography is proposed in this work. Because of the low quantities of pharmaceuticals in water and wastewater, pre-treatment is necessary before analysis. Solid-phase extraction (SPE) is one of the widely used techniques for analyte enrichment. In addition, this SPE process is also used to remove some potential background interfering compounds in wastewater [15].

Some of the commonly used techniques for sample extraction in water are liquid-liquid extraction [15, 16] and solid-phase extraction [17, 18]. Many polar analytes are often partly soluble in water, and good recoveries are difficult to obtain when extracted by organic solvents, so solid-phase extraction is used more often with polar analytes nowadays [19]. The advantage of being able to trap polar analytes and can be used to remove some potential background interfering compounds in wastewater, the solid-phase extraction technique will be used in this study.

2. MATERIALS AND METHODS

2.1. Materials and reagents

2.1.1. Chemicals

All solvents were HPLC grade, and all reagents were analytical grade. Paracetamol (99 %) was purchased from Sigma-Aldrich (USA). Diclofenac (99.9 %) was purchased from HPLC standards GmbH (Germany). HPLC-grade solvents such as methanol (> 99 %), acetonitrile (> 99.9 %), acetone (> 99.9 %), and ethanol (> 99.8 %) were purchased from Merck (Germany). Hydrochloric acid (37 %), citric acid (99 %), and sodium phosphate monobasic NaH_2PO_4 (> 99 %) were purchased from Merck (Germany). All solvents and solutions were filtered through a membrane filter or filtration units 0.45 µm and degassed before use.

2.1.2. Stock and standard solutions

Stock solutions (100 mg/ml) of DIC and PAR were prepared by dissolving 10 mg of standard DIC and PAR in a 100 ml volumetric flask with the methanol. Solutions were appropriately diluted with the mobile phase to obtain a mixed standard solution containing 10.00 µg/ml DIC and 10.00 µg/ml PAR.

2.2. Sample preparation and optimization of the SPE production

2.2.1. Stock and standard solutions

The study was conducted on raw and treated wastewater samples from a membrane bioreactor (MBR) of the wastewater treatment system. All samples were collected and kept in dark glass bottles. Within 2 hours of sample collection, the samples were filtered using a 0.45 µm membrane glass fiber filter (Whatman Mainstone, UK). To decrease biological activity, acidify the sample with 0.1 % HCl to pH of 2. Samples were stored in the dark at 4 °C until extraction, which usually happened 48 hours after collection.

2.2.2. Solid-phase extraction

The solid-phase extraction technique (SPE) is optimized using standard solutions to achieve high extraction efficiency for the target compounds. Before sample extraction, the SPE cartridge was activated with 5 ml of methanol and 5 ml of deionized water. Then, the sample was run through a cartridge at an appropriate flow rate (1.0, 4.0, 7.0, and 10.0 ml/min), and the solid phase was vacuum dried for 5 min. To achieve the highest possible concentration factor with minimal use of organic solvents (methanol, ethanol, and acetonitrile), the volumes of the solvent varied (1.0, 3.0, 5.0, 7.0, and 10.0 ml) with a flow rate of 1 ml/min were investigated to provide optimal conditions for analyte elution. Next, the solvent elution was evaporated, and the samples were re-dissolved in a 1 ml solution of the mobile phase. Then, the samples were filtered through Whatman (0.45 µm) syringe filter before injecting the samples into the HPLC instrument for quantification.

2.3. Equipment and separation conditions

HPLC Chromatography System: The HPLC chromatography system used in this study was a Shimadzu system, which included a controller (SCL-10A SP), high-pressure pump (LC-10A SP), sample injection unit (SIL-10AF), photodiode array detector -PDA detector (SPD-M20A), vacuum degasser (DGU-14A), heater (HIC-10A), and HSR C18 reversed-phase chromatography column (250 mm × 4.6 mm; 5 µm). The data were recorded using Shimadzu LCsolution software.

Sample Preparation: The water samples were prepared by filtering through a 0.45 µm syringe filter to remove any particulate matter. Solid phase extraction (SPE) was then performed using column solid-phase extraction C18 – Enviro Clean® SPE Cartridges size 500 mg/6 ml with optimized conditions described in section 2.2.2. The eluate was collected and evaporated to dryness and the residue was reconstituted in 1 ml of the mobile phase.

Other equipment used in the study included a UV-Vis spectrophotometer (Lambda 35 – Perkin Elmer), centrifuge (Centrifuge Combi 514R – Korea, Centurion Scientific K2015 – UK), solid-phase extraction device, column solid-phase extraction C18 – Enviro Clean® SPE Cartridges size 500 mg/6 ml, and a centrifuge (Centrifuge Combi 514R – Korea, United Chemical Technologies- UTC, USA).

UV-Vis Spectrophotometer: A Lambda 35 UV-Vis spectrometer (Perkin Elmer) was used to investigate the maximum absorption wavelength of the pharmaceutical.

Preliminary Testing: Prior to the actual experiments, preliminary testing was conducted to identify the optimal conditions for effective pharmaceutical analysis of the water samples. Several key factors that influence the chromatographic analysis process were investigated, including pH (3 - 8), the volume ratio of organic solvents and buffer solutions in the mobile phase (20 - 80 %), the choice of organic solvents (methanol, acetonitrile, ethanol), and the flow rate (0.4, 0.6, 0.8, 1.0, and 1.2 ml/min) on the HPLC system.

2.4. Validation of the method

After optimizing the procedure of simultaneous DIC and PAR analysis on HPLC systems, the system compatibility, linear range, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) of the technique were determined.

3. RESULTS AND DISCUSSION

3.1. Simultaneous determination of PAR and DIC in wastewater

SPE was optimized using a standard solution (0.1 µg/ml) to obtain high extraction efficiency for the analytes. The optimized parameters are the injected speed into the column of the extracted sample, the eluent solvent, and the volume of the eluate. The results of the survey were shown in Table 1.

The different solvent condition was investigated for the compounds that were adsorbed on the SPE cartridge. The three elution solvents under investigation were methanol, acetone nitrile, and ethanol. Methanol provided the optimum elution conditions, with recoveries for DIC and PAR of 98 % and 95 %, respectively.

Table 1. Investigation of extraction conditions in PAR and DIC in wastewater samples.

Extraction conditions		Recovery (%)	
		DIC	PAR
Elution	MeOH	98.54	95.23
	ACN	67.56	56.12
	EtOH	90.14	87.23
Flow rate for extraction (ml/min)	1	67.56	56.12
	4	90.14	87.23
	7	97.82	98.24
	10	97.92	98.04
Volume of eluent solution (ml)	1	78.12	73.24
	3	96.24	95.13
	5	97.82	98.24
	7	97.34	96.45
	10	98.14	97.89

When the sample injection speed through the column is from 1 to 10 ml/min, the survey results showed that the recovery did not change significantly with the flow rate between 4 and 10 ml/min. To achieve the best compromise between extraction efficiency and time, a flow rate of 7.0 ml/min was selected.

The elution solvent volume was also investigated in the range from 1 ml to 10 ml of MeOH solution. The survey results showed that the recovery did not change significantly in the range from 3 ml to 10 ml and achieved greater than 95 %. Therefore, 5.0 ml of methanol was chosen as the optimal condition for the extraction sample.

2.4. Study on the optimum condition for simultaneous determination of PAR and DIC by HPLC

UV-Vis spectra (Fig. 1) of solutions containing DIC and PAR with wavelengths ranging from 200 to 600 nm reveal that DIC has a maximum absorption peak at 276 nm while PAR has a maximum absorption peak at 243 nm. To achieve a good signal and high sensitivity, a PDA was employed to detect simultaneous detection of DIC and PAR antibiotics. Therefore, a wavelength of 243 nm was utilized for PAR quantification, and that of 276 nm was used for DIC quantification.

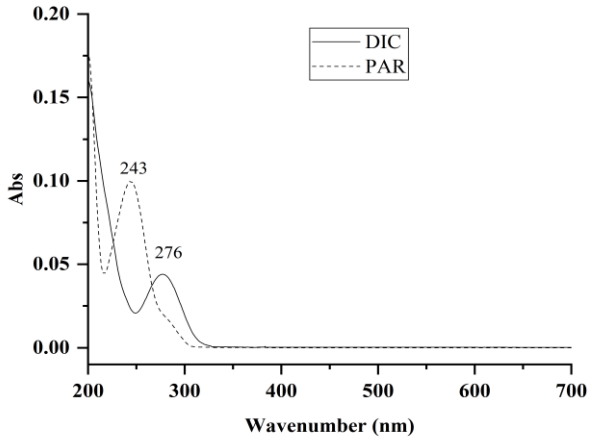

Figure 1. UV-Vis absorption spectra of DIC and PAR.

Preliminary experiments were performed to determine the main variables affecting the chromatographic separation, such as pH, organic solvents, the volume ratio of organic solvents, and buffer solutions in the mobile phase.

Different mobile phases with different compositions and polarity were tested to investigate chromatographic separation, including sodium dihydrogen phosphate-methanol buffer, sodium dihydrogen phosphate-acetonitrile buffer, and sodium dihydrogen phosphate-ethanol buffer. When using buffer sodium dihydrogen phosphate-acetonitrile, the signals of the DIC and PAR analytes were separated. In addition, the effect of the sodium dihydrogen phosphate-acetonitrile buffer ratio was also investigated to improve resolution were shown in Figure 2.

The maximum resolution was obtained using the proportional sodium dihydrogen phosphate-acetonitrile buffer (30:70, v/v). The strength of the buffer had a significant effect on the separation, so the type and concentration of the buffer solution were optimized.

Concentrations of sodium dihydrogen phosphate of 0.02 M, 0.1 M, and 0.2 M were used to investigate the suitable buffer concentration. During the experiment, the chromatograms were observed to be like these three buffer concentrations. Therefore, the lowest sodium dihydrogen phosphate concentration of 0.02 M was used.

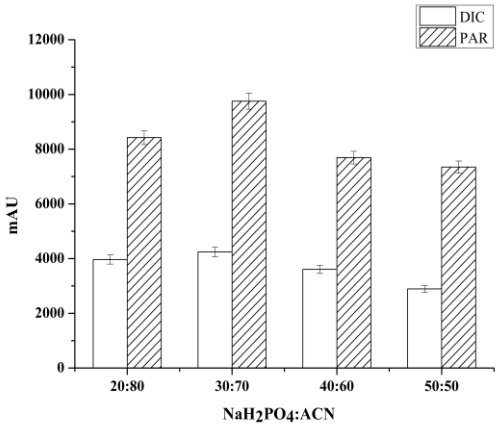

Figure 2. The sodium dihydrogen phosphate-acetonitrile buffer ratio in the mobile phase.

The effect of pH on the separation was tested by using buffers of different pH values (3 - 6). It was found that the change in pH improved the peak shape and decreased the tailing of the peaks. In this study, pH 5 showed good absorption, peak shapes, and the best chromatographic resolution between PAR and DIC, so phosphate buffer at pH 5 was selected for analysis.

Table 2. Optimal parameters for extraction and analysis.

Sample extraction conditions		Chromatographic conditions	
Extraction SPE	C18 Cartridges (500 mg, 6 ml)	Column	HSR C18 (250 mm × 4.6 mm; 5 µm)
Sample loading	5 - 10 ml/min	Detector	PDA
Drying	5 min	Wavelength	PAR: 243 nm DIC: 276 nm
Elution	5 ml Methanol	Mobile phase	Acetonitrile: 0.02M NaH_2PO4, pH = 5 (70:30)
Rate flow	1 ml/min	Rate flow	1 ml/min
Evaporation	Under N_2 flux until dryness Reconstitute in 1 ml of the mobile phase	Injection volume	20 µl

The flow rates of the mobile phase of 0.6, 0.8, 1.0, 1.2, and 1.5 ml/min were studied. The result showed that the retention time and the peak area decreased as the flow rate increased. Short retention time in the case of matrix samples as wastewater is susceptible to the effects of impurities, but long retention time takes a long time to analyze. Therefore, to decrease the detection limit for the selected flow rate method was 1.0 ml/min. The survey findings and the parameters chosen for simultaneous determination of the presence of DIC and PAR in

wastewater were shown in Table 2. With optimal conditions obtained, a typical chromatogram showing the separation of peaks of PAR and DIC was depicted in Figure 3.

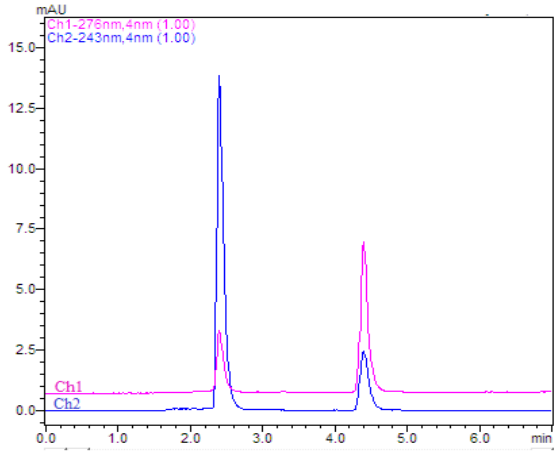

Figure 3. HPLC chromatogram of peaks separation of PAR and DIC.

2.5. Validation of the method

3.3.1. System suitability

The system suitability was checked by five replicate injections of the standard solution of a mixture containing 0.1 µg/ml PAR and 0.1 µg/ml DIC. 0.1 µg/ml. The system was judged acceptable for usage since the tailing factor for both medications was less than 1.5, and the resolution was more than 2. The chromatographic parameters of the drug were reported in Table 3.

Table 3. The chromatographic parameters for the assayed drugs.

Drug	R_t	R_s	N	T_f
PAR	2.39		2399970	1.497
DIC	4.39	6.51	5101038	1.286

R_t: retention time; R_s: resolution; N: number of theoretical plates and T_f: tailing factor.

3.3.2. Linearity, LOD, and LOQ

Table 4. Linearity data, LOD, and LOQ of PAR and DIC.

	PAR	DIC
Range (µg/ml)	0.01-1	0.02-1
Equation	y = 91659.52x + 687.04	y = 40481.57x + 104.12
Correlation coefficient (R^2)	0.9999	0.9998
LOD (µg/ml)	0.003	0.006
LOQ (µg/ml)	0.01	0.02

Set up a calibration curve for PAR and DIC analysis with the above-mentioned optimal conditions by plotting the peak area as a function of PAR and DIC concentrations (in µg/ml). Table 4 included linearity data, LOD, and LOQ of PAR and DIC. The LOD and LOQ were determined based on the signal-to-noise ratio using the analytical response of 3 and 10 times for the background noise, respectively.

3.3.3. Precision and accuracy

The intra-day precision was evaluated by injecting six replicates of three standard solutions covering low, medium, and high concentration levels (0.04, 0.2, and 0.8 µg/ml) on a single day. The inter-day precision of the proposed method was performed by chromatographing standard solutions of the same concentration levels analyzed in triplicate on each of the three consecutive days. The accuracy was then calculated as the percentage of each drug recovered by the experiment. The mean value of the concentration, % relative standard deviation (% RSD), and recoveries were summarized in Table 5. The results demonstrated that the analyses provided strong repeatability with RSD_R (1.10 - 3.58 %), RSD_r (1.33 - 4.11 %), and high recovery (> 98 %). As a result, the analytical technique had excellent accuracy under the AOAC standards.

Table 5. Accuracy and precision data of PAR and DIC.

Drug	Concentration (µg/ml)	Intra-day (n = 6)			Inter-day (n = 9)		
		Mean ± SD (µg/ml)	RSD_R (%)	Recovery (%)	Mean ± SD (µg/ml)	RSD_r (%)	Recovery (%)
PAR	0.04	0.040 ± 0.001	1.35	98.65	0.040 ± 0.001	1.68	98.32
	0.2	0.199 ± 0.002	0.50	99.50	0.198 ± 0.002	1.01	99.22
	0.8	0.798 ± 0.003	0.40	99.75	0.796 ± 0.004	0.54	99.58
DIC	0.04	0.040 ± 0.001	1.57	98.43	0.040 ± 0.001	1.89	98.11
	0.2	0.202 ± 0.003	1.49	101.03	0.199 ± 0.002	1.05	99.23
	0.8	0.795 ± 0.004	0.48	98.89	0.792 ± 0.004	0.60	98.40

2.6. Application of the method for simultaneous determination of PAR and DIC in wastewater

The validated method was applied for the simultaneous determination of PAR and DIC in the inlet (IW) and outlet (OW) wastewater samples from the MBR of the wastewater treatment system. Obtained results were given in Table 6. The results of PAR and DIC in wastewater showed that the analyzed samples had good repeatability with RSD < 7 %.

Table 6. Analysis results of wastewater.

Sample	Parameter	The amount found (µg/ml)	RSD % (n = 3)
IW	PAR	0.0053 ± 0.0001	2.45
	DIC	0.0110 ± 0.0002	1.91
OW	PAR	0.0016 ± 0.0001	5.06
	DIC	0.0027 ± 0.0002	6.67

4. CONCLUSIONS

The proposed extraction and determination method demonstrated the ability to extract and simultaneously determine PAR and DIC in wastewater by HPLC using a mobile phase Acetonitrile: 0.02M NaH_2PO_4, pH = 5 (70:30), and to extract the analytes in a single procedure using a C18 cartridge and methanol as eluent. The approach was selective, simple, and quick, with sufficient LOD and LOQ, and it can be an essential tool for laboratories dedicated to medical analysis.

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Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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