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9-Fluorenylmethoxycarbonyl Chloride (FMOC-Cl) as a Potential Precolumn Derivatization Reagent for the Analysis of PPCPs and EDCs using HPLC-DAD

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In this study, a simple precolumn derivatization method using 9-fluorenylmethoxy-carbonyl chloride (FMOC-Cl) was introduced to enable the simultaneous determination of 13 selected pharmaceuticals and personal care products (PPCPs) and endocrine disrupting chemicals (EDCs) using HPLC coupled with diode array detector. The selected compounds included both derivatizable (bisphenol A, methylparaben, ethylparaben, propylparaben, butylparaben, benzylparaben, atenolol and metoprolol) and non-derivatizable (dimethyl phthalate, diethyl phthalate, dipropyl phthalate, phenazone and N, N-diethyl-m-toluamide) compounds. The derivatization was performed in a water-acetonitrile mixture (1:1 (v/v)) at room temperature with pH 10 borate buffer as the catalyst. After derivatization, hydrochloric acid was added to the mixture to stabilize the FMOC derivatives. Following derivatization, the separation of the selected compounds was largely improved, and stable FMOC derivatives also enabled the analysis to be performed using automated procedures. The results also showed that all the derivatizable compounds exhibited good linearity and high correlation coefficients (0.9995 to 0.9998).

Key words: PPCPs, EDCs, environmental analysis, FMOC, derivatization

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The presence of pharmaceuticals and personal care products (PPCPs) and endocrine disrupting chemicals (EDCs) in the environment have drawn much attention among the scientific community as a result of their potential to cause undesirable ecological and human health effects [1]. PPCPs and EDCs often occur at trace levels ranging from ng L^{-1} to hundreds of µg L^{-1}. Thus, in order to monitor and understand the behavior of these compounds in the aquatic environment, an analytical method with higher sensitivity and resolution is required.

HPLC is one of the most commonly used detection methods in analytical chemistry [2]. Most of the sample extracts obtained from the environment frequently interfere with other compounds, which may overlap with the signals of targeted analytes. The frequently reported detection method used for the determination of PPCPs and EDCs in environmental samples is the high-performance liquid chromatography coupled with tandem mass spectrometry (LCMS/MS) [3,4]. These instruments are very costly and consequently not widely distributed, especially in developing countries. In contrast, the HPLC system with a diode array detector (DAD) is more common in most of the laboratories. The main objective of this study was to develop a derivatization method to enhance the sensitivity and separation of PPCPs and EDCs during analysis using HPLC-DAD.

9-Fluorenylmethoxycarbonyl chloride (FMOC-Cl) is one of the most commonly used fluorescence labeling reagents for the analysis of amino acids. It does not only react with compounds containing amino group but also with compounds that contain hydroxyl group [5-7] (Figure 1). Thus, it can be a potential derivatization reagent in the analysis of PPCPs and EDCs that contain hydroxyl group. Based on our literature search, application of FMOC-Cl in the environmental analysis of PPCPs and EDCs has not been reported. In this experiment, FMOC-Cl was applied as a precolumn derivatization reagent for selected PPCPs and EDCs by using HPLC-DAD. The selected compounds were bisphenol A (BPA), N,N-diethyl-m-toluamide (DEET), methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), butylparaben (BuP), benzylparaben (BzP), dimethyl phthalate (DMP), diethyl phthalate (DEP), dipropyl phthalate (DPP), phenazone (Phz), atenolol (Atnl) and metoprolol (Mtpl).

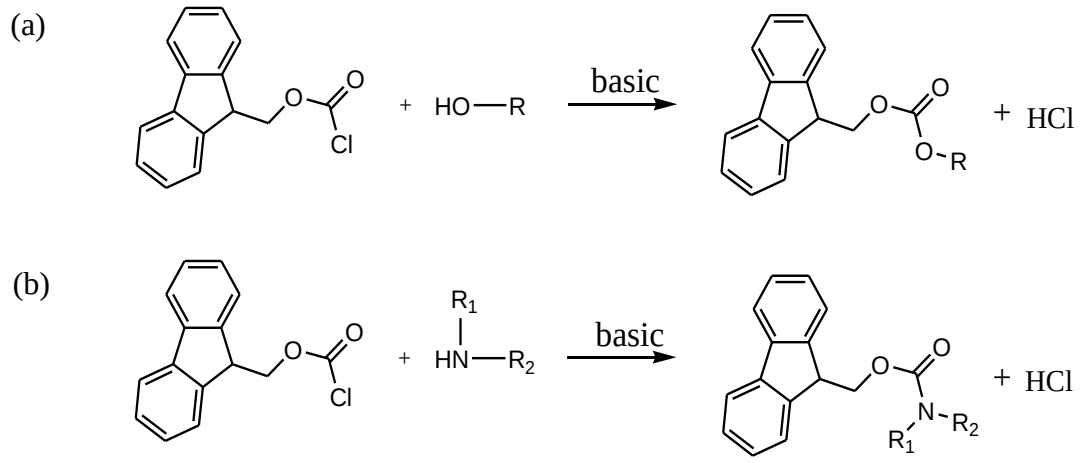


Figure 1. Derivatization reaction of FMOC-Cl with molecules that contain (a) hydroxyl and (b) amine groups.

This complex mixture of PPCPs and EDCs consisted of both derivatizable (with hydroxyl and amine as the functional groups) and non-derivatizable compounds.

EXPERIMENTAL

Chemicals and reagents

BPA, DEET, DMP, DEP, DPP, Atnl, Mtpl, 9-fluorenylmethanol (FMOC-OH) and trifluoroacetic acid (TFA) were obtained from Sigma-Aldrich (USA). MeP, EtP, PrP, BuP, BzP, FMOC-Cl, boric acid, and sodium hydroxide were purchased from Fluka (Germany). HPLC grade solvent and hydrochloric acid were purchased from Merck (Germany). The pH 10 borate buffer solution (0.5 M) was prepared by dissolving the appropriate amount of boric acid in water, and the pH was adjusted by using sodium hydroxide solution. 10 mM FMOC-Cl in acetonitrile solution was used throughout the experiment. A stock solution containing 100 mg L^{-1} of each compound was prepared in acetonitrile and stored at 4 ºC before use.

Instrumentation and analytical conditions

The analyses were carried out using a high-performance liquid chromatography system consisting of an LC-20AT pump, an SPD-M20A diode array detector, a SIL-20AHT autosampler, a CTO-20AC column oven and a CBM-20A communication bus module (Shimadzu, Japan). A reversed-phase Chromolith RP-18 monolithic column (100 mm × 4.6 mm i.d.; Merck, Germany) was used for separation. Ultrapure deionized water with 0.1% TFA (Solvent A) and acetonitrile (Solvent B) was used as mobile phase. The solvent gradient is presented in Table 1. The injection volume was 20 µL and the flow rate was constant at 2.0 ml min^{-1}, with the column temperature set at 30°C. A 5 min equilibration using 100% A was carried out between sample injections. The wavelength used for detection and quantitation was 230 nm. LC solution software was used to collect and analyze the chromatographic data.

Table 1. Mobile phase gradient of the HPLC method.

Time (min)	% of solvent A	% of solvent B
Initial	100	0
2.0	77	23
10.5	35	65
11.5	35	65
17.5	0	100
19.5	0	100

Derivatization procedure

Precolumn derivatization was performed by adding 25 μL of mixture solutions and 100 μL of FMOC-Cl solution into 2 mL vials containing 475 μL of acetonitrile, 325 μL of deionized water and 75 μL of borate buffer. The derivatization reaction was maintained at room temperature for 1 h before instrumental analysis. All experiments were performed in triplicates.

RESULTS AND DISCUSSION

Optimization of derivatization procedure

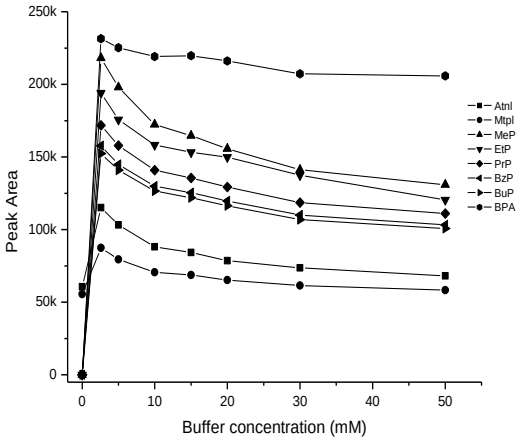
At room temperature, the variation of peak area of the derivatizable compounds was found to be highly influenced by the concentration of borate buffer, the concentration of FMOC-Cl, reaction time and medium of reaction (Figure 2). A mixture of 2.5 mg L^{-1} of each compound was selected to study the influence of the parameters mentioned above.

All the derivatizable compounds were found to react with FMOC-Cl under alkaline conditions. Compounds with hydroxyl group were found to be less reactive toward FMOC-Cl due to the less nucleophilic nature of hydroxyl group compared to amine group [8]. Thus, basic media are often required to catalyze the reaction. In this study, borate buffer with pH 10 was selected to provide the basic media. The peak areas were found to increase with increasing buffer concentration up to 2.5 mM. However, the peak areas decreased at higher buffer concentration (Figure 2(a)). These results might due to FMOC-Cl decomposition and the reduced amount of available FMOC-Cl for derivatization.

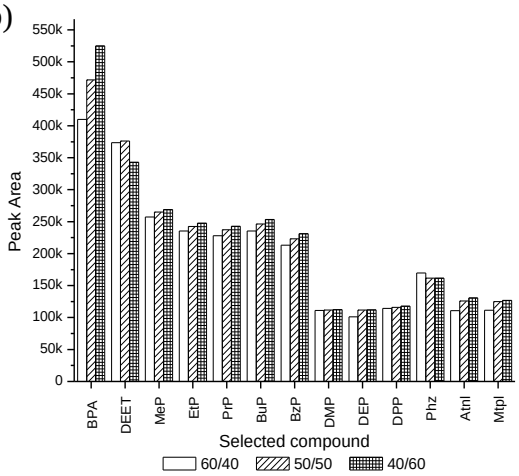
Appropriate amounts of acetonitrile and water are desired in order to dissolve FMOC-Cl and borate buffer. The peak areas for most of the selected compounds were found to increase with the amount of water (Figure 2(b)). The peak areas of the samples with a high content of organic solvent were found to be diluted significantly. In order to maintain the sample volume for better sensitivity, the medium which consisted of acetonitrile and water with a ratio of 50:50 (v/v) was selected. The ratio of acetonitrile and water of 40:60 (v/v) which had better response was not selected for the derivatization procedure because of the occurrence of precipitation due to the low solubility of FMOC derivatives in water.

During derivatization, FMOC-Cl did not only react with the interested analytes but also decomposed to form by-products such as 9-flurenylmethanol and 9-fluorenylmethyl hydrogen carbonate [9]. Consequently, excess FMOC-Cl must be added to the samples. In order to reduce the interference from the by-products during analysis, their formation has to be reduced by reducing the amount of initial FMOC-Cl concentration. In the experiment, the results indicated that the peak areas of derivatizable compounds reached the maximum at 1.2 mM of FMOC-Cl and the peak areas almost remained unchanged when the concentration of FMOC was further increased to 2.0 mM (Figure 2(c)). For better detection range, FMOC-Cl with the concentration of 2.0 mM was selected.

(a)



(b)



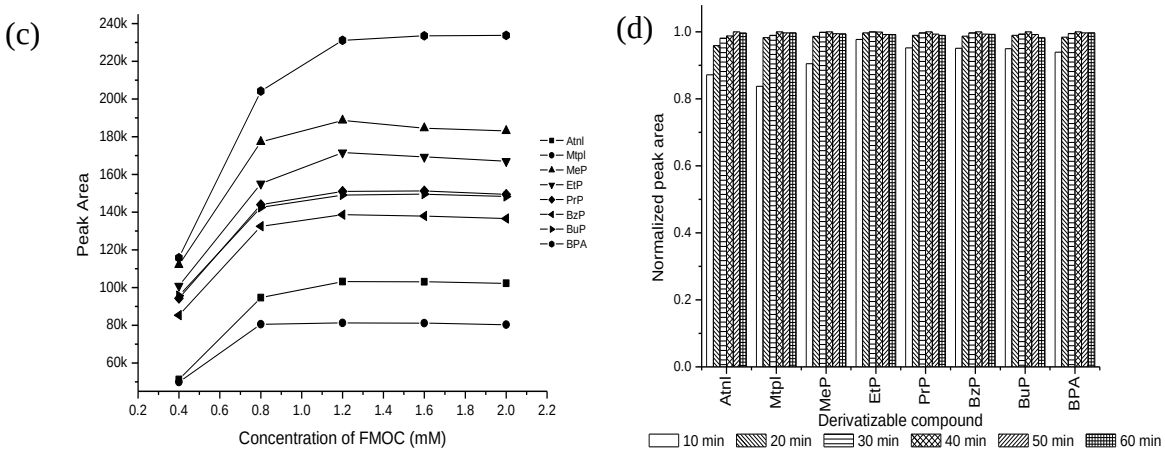


Figure 2. Effects of (a) buffer concentration, (b) composition of reaction medium (acetonitrile to water ratio), (c) FMOC-Cl concentration and (d) time of derivatization on the peak areas of derivatizable compounds.

FMOC derivatives were unstable under basic conditions. As shown in Figure 2(d), the peak areas for most of the FMOC derivatives decreased after reaching the optimum at 40 min. Consequently, acidification of the FMOC derivatives using 100 µL of 0.1 M of HCl was performed at 40 min, and the FMOC derivatives were found to be stable under such conditions for at least 24 h. A produced FMOC derivative with high stability enables the analysis to be performed using autosampler.

Based on the results, the optimal derivatization conditions were as follows: compounds were first dissolved in acetonitrile followed by the addition of deionized water, 2.5 mM of borate buffer (pH 10) and 2.0 mM of FMOC-Cl solution. After 40 min, 0.1 M of HCl was added. The total volume of the mixture was 1000 µL with the ratio of acetonitrile to water equaled to 6:4 (v/v) before the addition of 100 µL of 0.1 M of HCl. The linearity of this developed method was tested for all the derivatizable compounds (Table 2). All the derivatizable compounds exhibited good linearity within 0.2 – 5.0 mg L^{-1} and 0.2 – 2.5 mg L^{-1} for parabens and BPA, respectively. Obtained correlation coefficients were ranged from 0.9995 to 0.9998. Intraday and interday repeatability of the derivatization procedure on the derivatizable compounds expressed as relative standard deviations (%R.S.D.), was as low as 3% and 6%, respectively (Table 2). Thus, the developed derivatization procedure can give good reproducibility within the linearity range.

Table 2. Linear range, linearity, intraday and interday repeatability for the developed derivatization method.

Compound	Linear range (mg L^{-1})	Linearity (R^2)	Intraday Repeatability RSD[a] (%, n = 3)			Interday Repeatability RSD[b] (%, n = 6)		
			0.2 mg L^{-1}	2.5 mg L^{-1}	5.0 mg L^{-1}	0.2 mg L^{-1}	2.5 mg L^{-1}	5.0 mg L^{-1}
Atnl	0.2 – 5.0	0.9996	1.8	1.5	1.0	4.9	5.2	4.0
Mtpl	0.2 – 5.0	0.9997	2.5	1.7	1.4	4.7	2.9	5.8
MeP	0.2 – 5.0	0.9995	1.7	1.2	1.7	4.9	5.5	1.7
EtP	0.2 – 5.0	0.9996	1.9	1.2	1.3	5.0	4.5	1.5
PrP	0.2 – 5.0	0.9995	1.9	0.7	1.7	5.7	2.5	3.3
BuP	0.2 – 5.0	0.9997	1.3	1.2	1.8	5.7	2.4	4.4
BzP	0.2 – 5.0	0.9996	1.6	0.5	2.0	5.3	3.4	3.7
BPA	0.2 – 2.5	0.9995	1.5	1.8	~	4.8	5.4	~

~ out of linearity range

Chromatographic Separation enhancement

Figure 3 shows the HPLC chromatograms for the analytical separation of the studied compounds before (Figure 3(a)) and after (Figure 3(b)) derivatization, at the same analytical conditions. Figure 3(b) shows that the compounds were well separated after derivatization. Due to the non-polar behavior of FMOC derivatives, the retention times of the derivatizable compounds were delayed after derivatization. The retention times for non-derivatizable compounds, as expected, remained unchanged. Also, the elution order for the derivatizable compounds was changed from Atnl, Mtpl, MeP, EtP, PrP, BPA, BuP and BzP to Atnl, Mtpl, MeP, EtP, PrP, BzP, BuP, and BPA. Peaks I and II were attributed to derivatization by-products of FMOC. Peaks I and III were identified as 9-fluorenylmethanol and excess FMOC-Cl, respectively. Peak II is suspected to be 9-fluorenylmethyl hydrogen carbonate. Beside the separation, the detection sensitivity for derivatizable compounds has been largely improved (Figure 3), as it can be observed from the intensity of the peaks.

CONCLUSION

In conclusion, this study has shown that FMOC-Cl is a good derivatization reagent for separation enhancement in the determination of PPCPs and EDCs using HPLC-DAD. The developed derivatization procedure was proved to be a simple, reproducible and able to enhance the separation and detection sensitivity in HPLC analysis.

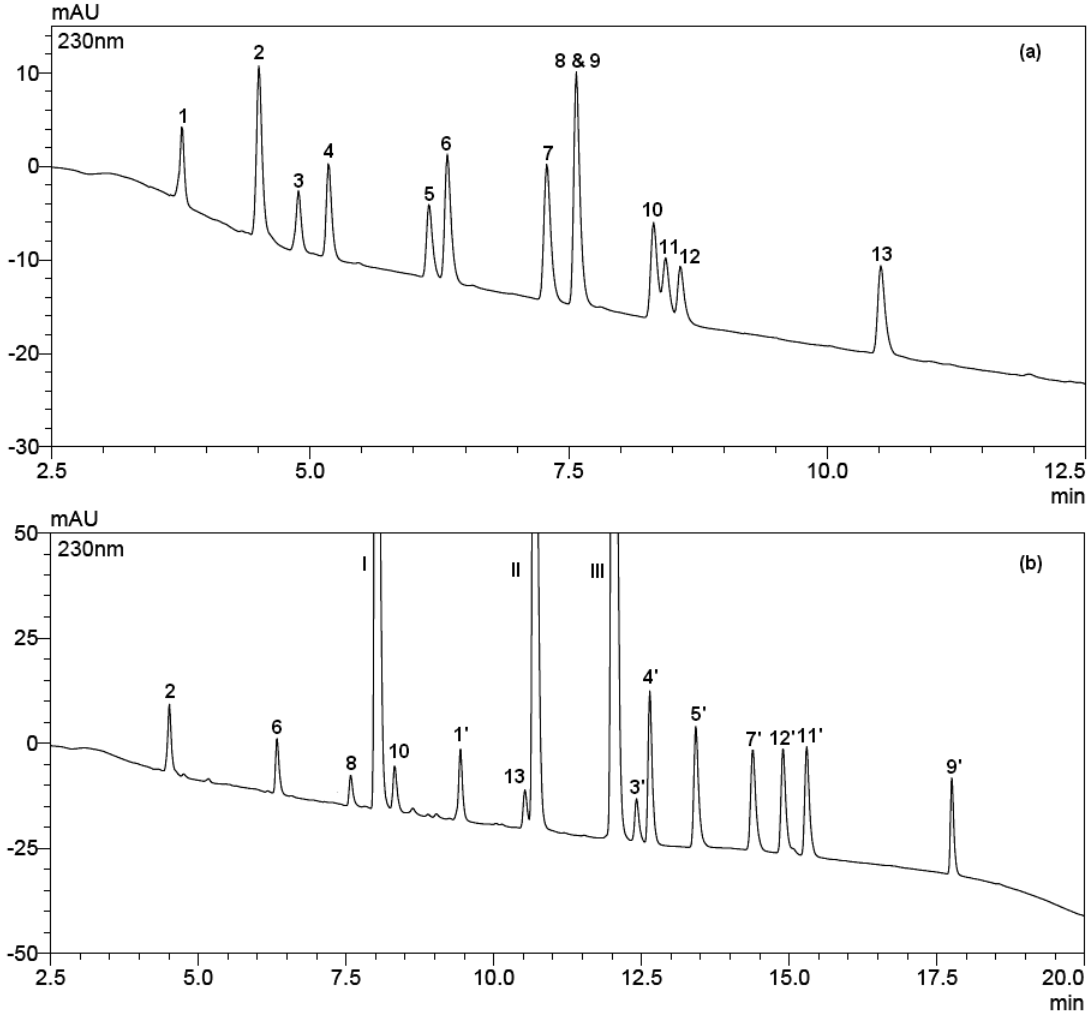


Figure 3. HPLC chromatograms of standard mixture solution (a) before and (b) after derivatization, detected at 230 nm. [(a) Peaks: (1) Atnl; (2) Phz; (3) Mtpl; (4) MeP; (5) EtP; (6) DMP; (7) PrP; (8) DEET; (9) BPA; (10) DEP; (11) BuP; (12) BzP and (13) DPP. (b) Peaks: (1') FMOC-Atnl; (2) Phz; (3') FMOC- Mtpl; (4') FMOC- MeP; (5') FMOC-EtP; (6) DMP; (7') FMOC-PrP; (8') DEET; (9') FMOC-BPA; (10) DEP; (11') FMOC-BuP; (12') FMOC-BzP; (13) DPP; (I) and (II) Derivatization by-products of FMOC-Cl and (III) FMOC-Cl].

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