Analysis of phenols by high-performance liquid chromatography with pre-column derivatization by 2-(9-carbazole)-ethyl-chloroformate

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Abstract
2-(9-Carbazole)-ethyl-chloroformate (CEOC), a novel pre-column fluorescence labeling reagent, has been synthesized and applied for the derivatization of phenols. Taken phenol, p-chlorophenol, 2,5-dimethylphenol, 2,4-dichlorophenol and 1,4-dihydroxybenzene as testing standards, the effects of derivatization conditions, such as pH of borate buffer, reaction time and fluorescent tagging reagent concentration, have been systematically studied. Under the optimized conditions, CEOC reacts readily with the phenols to form stable derivatives with excitation and emission wavelengths, respectively, at 293 and 360 nm. The single step derivatization reaction could be finished within 20 min even at room temperature. Such a method has been successfully applied to the analysis of phenols in printing ink by high-performance liquid chromatography.

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1. Introduction

Phenol and substituted phenols belong to a family of compounds especially harmful for the environment, owing to their toxicity and carcinogenic effect [1]. These compounds can be the source of serious health hazards if released into the environment through accidental spillage or poor disposal [2–4]. Recently, the determination of such compounds has been paid much attention, and a variety of analytical methods have been reported. The hyphenation of gas chromatography (GC) and mass spectrometry (MS) has proven powerful for phenolic compounds assay [5,6], but the cost is generally high. For only GC based analysis, the poor polarity and insufficient sensitivity of phenols are troublesome [7–10] so that chemical derivatization, such as silylation [11,12] and esterification [13] is necessary, however, on the consumption of large amount of samples. Direct detection of these compounds by high-performance liquid chromatography (HPLC) is also difficult because they lack appropriate spectroscopic properties [14–17]. In addition, because of double retention mechanism of phenols on C18 column, the peak tailing is very serious with acetonitrile and water as mobile phase [18]. Therefore, suitable derivatization is also required [19–22]. Derivatization is time-consuming, but the derivatives can sometimes be determined at more selective wavelengths and may be easier to analyze by LC because of higher retention or better peak shapes. However, only a few labeling reagents have been used for this purpose, such as 2-methoxy-4-(2-phthalimidinyl)-phenylsulfonyl chloride [19], 4-(4,5-diphenyl-imidazol-2-yl)benzoyl chloride [20,21], 4-(N-phthalimidinyl)benzenesulfonyl chloride [22], anthraquinone-2-sulfonyl chloride [23] and dansyl chloride [5-(dimethylamino)-1-naphthalenesulfonyl chloride] (Dns-CI) [24].

We all know that Dns-CI is a well-known commercial fluorescence reagent used for the derivatization of amino acids, amines and phenols. Kwakman et al. [25] used Dns-CI to derivatize phenols after pre-concentration of the phenols with C18 cartridge to get high detection sensitivity, but the proce-
dure needed to remove the excess of derivatizing reagent with an amino solid phase extraction (SPE) column before HPLC analysis. In addition, Dns-Cl and its derivatives are usually unstable to light, requiring precautions during the analytical procedure [23].

Recently, we have synthesized a new fluorescent labeling reagent, 2-(9-carbazole)-ethyl chloroformate (CEOC), for the analysis of amino acids and aromatic amines by HPLC [26,27]. In this study, to the best of our knowledge, for the first time, CEOC was applied for the derivatization of phenolic compounds. With five phenols as testing compounds, the effects of reaction conditions have been studied, and the linearity, detection limit and the precision of this method have been obtained. The proposed method has also been successfully applied to the determination of phenols in printing ink. All these results demonstrate the advantages of such a method, such as mild reaction condition, fast derivatization speed, good stability of derivatives and high detection sensitivity.

2. Experimental

2.1. Instrumentation

HPLC experiments were performed on a system with Jasco PU-1580 intelligent pump equipped with a Jasco LG-1580-54 quaternary gradient unit, a Jasco DG-1580-04 4-line degasser (Jasco Co, Tokyo, Japan), a Model 7125 injection valve (Rheodyne, Cotati, CA, USA), a 250 mm × 4.6 mm i.d. (5 µm) Hypersil BDS C18 column (Elite Analytical Instrument Ltd., Dalian, China) and a Jasco FP-2020 intelligent fluorescence detector (Jasco Co., Tokyo, Japan). The mobile phases were degassed in an ultrasonic bath for 15 min prior to use.

2.2. Chemicals

All reagents were of analytical grade unless otherwise stated. Phenols and boric acid were purchased from Shanghai Chemical Reagent Co. (Shanghai, China). Water was purified with a CLEAR (SG, Fahrenberg, Germany). CEOC was synthesized in our laboratory according to the previously reported method [26]. Printing ink was from EPSON TO38 for EPSON Stylus C43UX/SX printer (Beijing, China). HPLC-grade acetonitrile was purchased from Yuwang Chemical Reagent (Shandong, China). Borate buffer was prepared from the solution of boric acid (0.2 mol L⁻¹) and adjusted to pH 9.0 with 4 mol L⁻¹ sodium hydroxide solution.

2.3. Preparation of standard solutions

The derivatization reagent solution (1.0 × 10⁻³ mol L⁻¹) was prepared by dissolving 2.74 mg 2-(9-carbazole)-ethyl chloroformate in 10 mL of anhydrous HPLC-grade acetonitrile, which was dried with P₂O₅. Individual stock solutions of phenols were prepared in water with concentration of 1.0 × 10⁻³ mol L⁻¹. The standard phenols for HPLC analysis were obtained by diluting the corresponding stock solutions to the concentrations of 1.0 × 10⁻⁴ mol L⁻¹. When not used, all standards were stored at 4 °C.

2.4. Derivatization procedure

A 100 µL solution of phenols was put into a vial; then, 100 µL 0.2 mol L⁻¹ borate buffer (pH 9.0) and 100 µL of CEOC acetonitrile solutions were added. After 20 min reaction, 50 µL NH₃·H₂O (10%, w/w) was added to remove the excess derivatization reagent. A 30 µL volume of the
2.5. Preparation of practical sample

The solution of printing ink (50 g L\(^{-1}\)) was prepared by dissolving 0.5 mg printing ink in 10 mL of water. The extraction procedure of phenol in printing ink proceeded according to the previously described method \([28]\). A 500 μL solution of printing ink was placed in a screw-capped vial to which 2 mL chloroform was added. The vial was degassed in an ultrasonic bath for 5 min. A 1 mL volume of the extract solution in chloroform phase was dried with a nitrogen stream, dissolved to 500 μL with acetonitrile and then filtrated by a 0.45-μm filter membrane. The solution was derivatized with the above-mentioned procedure.

2.6. Chromatographic method

HPLC separation of CEOC derivatives was carried out on a Hypersil BDS C\(18\) column by a binary gradient elution. Eluent A was acetonitrile/water (20/80, v/v); eluent B was acetonitrile/water (95/5, v/v). The gradient condition used for...
the separation of phenol derivatives was as follows: 0–15 min: 35% A, 15–35 min: 35–0% A. The flow rate was constant at 1.0 mL/min and the column temperature was at room temperature. The fluorescence excitation and emission wavelengths were set, respectively, at 293 and 360 nm. Before each run, HPLC column was equilibrated with the initial mobile phase for 20 min.

3. Results and discussion

3.1. Fluorescence properties

Jasco FP-2020 intelligent fluorescence detector has a function of on-line wavelength scanning, with which the fluorescence spectra of CECO-derivatized phenol could be obtained. Fig. 2 shows the fluorescence spectra of CECO-derivatized phenol in acetonitrile. It can be seen that the maximum emission and excitation wavelengths were at 293 and 360 nm, respectively. Therefore, such two wavelengths were chosen in our following experiments.

3.2. Effect of pH of buffer on derivatization

For the derivatization, it has been found that the pH value of buffer has great effect on the yield and stability of derivatives. At a given concentration of CECO and reaction time, the influence of pH on the relative yield of the derivatized phenols was investigated within a range of 8.5–10.0. From Fig. 3a, it could be seen that the relationship is not linear. For 1,4-dihydroxybenzene, the yield decreases with pH, and the maximum could be obtained at pH 8.5. For other phenols, the reaction yields increase with the pH of buffers from 8.5 to 9.0, while decrease with the further increase of the pH of buffer. Accordingly, pH 9.0 is selected as the final buffer pH.

3.3. Effect of reaction time on derivatization

The optimum derivatization time has been investigated at ambient temperature, as shown in Fig. 3b. For 2,4-dichlorophenol and p-chlorophenol, the relative yields reach 100 and 96% within 5 min, respectively. The phenolic hydroxyls in these two phenols are more active than others. For phenol and 1,4-dihydroxybenzene, the relative yields reach 95% within 10 min and no obvious increase is observed after that. However, to achieve the maximum yield, the reaction time for 2,5-dimethylphenol is 20 min, which might be caused by the steric interference. Therefore, 20 min is sufficient for the simultaneous derivatization of all these phenols.

3.4. Effect of fluorescent tagging reagent concentration on derivatization

The effect of CECO concentration on the yields of derivatized phenols has been studied, as shown in Fig. 3c. The results indicate that when the molar reagent excess is over 6.4, the relative yields of derivatives reach the maximum. With as little as a 2-fold molar excess of derivatizing agent, the derivatization of 1,4-dihydroxybenzene was incomplete and resulted in monosubstituted derivatives. In the following experiments, 10-fold equivalent excess amount of the labeling reagent was taken so that all of phenols could be derivatized completely, especially for 1,4-dihydroxybenzene.

3.5. Analysis of derivatized phenols

Under the optimum derivatization conditions, five phenols were derivatized by CECO and analyzed by HPLC. Although there are some impurities of the synthesized CECO after 1 year, they are proven not to interfere the separation of derivatized phenols. In our experiment, baseline separation for five CECO-labeled phenols on a reversed-phase Hypersil BDS 

<table>
<thead>
<tr>
<th>Phenols</th>
<th>Peak area (R.S.D.%)</th>
<th>Retention time (R.S.D.%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>1.3</td>
<td>0.56</td>
</tr>
<tr>
<td>p-Chlorophenol</td>
<td>0.99</td>
<td>0.71</td>
</tr>
<tr>
<td>2,5-Dimethylphenol</td>
<td>1.3</td>
<td>0.76</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>5.7</td>
<td>0.81</td>
</tr>
<tr>
<td>1,4-Dihydroxybenzene</td>
<td>1.0</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Table 1

Repeatability of peak area and retention times for 57 pmol of each phenol (standard (n = 5))

From Table 2, we could see that good linear relationship between injection amount and peak area of derivatized phenols could be obtained. By further calculation, the detection limits of (signal-to-noise ratio = 3) are found at the level of 109.3–243.3 fmol, which demonstrates the high sensitivity of this method.

The analysis of phenol in printing ink is performed as a practical application for CECO. Fig. 4b shows a phenol
Fig. 4. Analysis of CEOC-labeled phenols: (a) 57 pmol of each phenol standard and (b) 0.73 pmol phenol in printing ink. Chromatographic conditions: column, 250 mm × 4.6 mm i.d. Hypersil BDS C18 5 μm; eluent (A) was acetonitrile/water (20/80, v/v); (B) acetonitrile/water (95/5, v/v); gradient conditions: 0–15 min: 35% A; 15–35 min: 35–0% A; flow rate: 1.0 mL/min; the column temperature: ambient temperature. Peaks: (1) phenol, (2) p-chlorophenol, (3) 2,5-dimethylphenol, (4) 2,4-dichlorophenol and (5) 1,4-dihydroxybenzene.

Table 2

<table>
<thead>
<tr>
<th>Phenols</th>
<th>Linear range (pmol)</th>
<th>R</th>
<th>Regression equation</th>
<th>LOD (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol</td>
<td>3.0–194.3</td>
<td>0.999</td>
<td>$y = 0.087 + 0.150x$</td>
<td>164.4</td>
</tr>
<tr>
<td>p-Chlorophenol</td>
<td>3.9–196.9</td>
<td>0.999</td>
<td>$y = 0.471 + 0.139x$</td>
<td>109.3</td>
</tr>
<tr>
<td>2,5-Dimethylphenol</td>
<td>4.2–207.50</td>
<td>0.999</td>
<td>$y = 0.392 + 0.0638x$</td>
<td>243.3</td>
</tr>
<tr>
<td>2,4-Dichlorophenol</td>
<td>6.1–182.0</td>
<td>0.997</td>
<td>$y = -1.380 + 0.122x$</td>
<td>174.1</td>
</tr>
<tr>
<td>1,4-Dihydroxybenzene</td>
<td>11.7–233.8</td>
<td>0.999</td>
<td>$y = 1.779 + 0.0764x$</td>
<td>120.3</td>
</tr>
</tbody>
</table>

* y: peak area (V s); x: injection amount (pmol).
* LOD: The ration of signal-to-noise = 3.

peak at 11.66 min. Although a number of additional CEOC-reactive impurities are observed, the peak identification is confirmed by comparing the retention time to that of the standard. Based on the relationship of injected amount and peak area of derivatized phenol, the concentration of phenol in printing ink can be calculated according to the diluent multiple of sample in the process of preparation and derivatization. It is corresponding to a level of 4.26 μg/mL. The result is similar to that of reference [28], which is at a level of 6.74 μg/mL.

4. Conclusions

It has been shown that 2-(9-carbazole)-ethyl-chloroformate is a sensitive and convenient pre-column derivatizing reagent for the determination of phenols by HPLC with fluorescence detection. Complete derivatization in basic medium at room temperature takes less than 20 min, and derivatives were stable for at least 1 week in neutral medium stored at 4 °C in a refrigerator. The derivatization procedure is proven rapid, simple and reproducible, making the method attractive for the analysis of phenols in environmental and toxicological investigations.

Acknowledgments

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