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Sensitive high-performance liquid chromatographic determination with fluorescence detection of phenol and chlorophenols with 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride as a labeling reagent

Mitsuhiro Wada, Shinobu Kinoshita, Yuko Itayama, Naotaka Kuroda, Kenichiro Nakashima*

School of Pharmaceutical Sciences, Nagasaki University, 1–14 Bunkyo-machi, Nagasaki 852–8521, Japan

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Abstract

A sensitive HPLC method for the determination of phenol and chlorophenols was developed. The fluorescence labeling reaction of phenols with 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) was completed in 30 min at 60°C. The separation of DIB-derivatives of five representative phenols, i.e., phenol, *o*-, *p*-chlorophenol, 2,4-dichlorophenol, 2,4,6-trichlorophenol, was achieved within 35 min with an ODS column using isocratic elution. The detection limits of these DIB derivatives at a signal-to-noise ratio (S/N) of 3 were in the range of 0.024 to 0.08 μ*M* (0.12–0.45 pmol/20 μl injection). Twelve kinds of DIB derivatives with phenols containing mono-, di-, tri-, tetra- and penta-chlorophenol were also well separated within 208 min by changing the elution conditions. The derivatives were stable for at least for 24 h when they were placed at room temperature in the dark. The proposed method was applied to the assay of human urine samples and free and total phenol were determined. The relative standard deviations (RSDs) of the proposed method for within and between-day assay were <7.0% and <14.2%, respectively. The average concentrations of free and total phenol found in urine ($n=6$) were 4.3±2.5 and 29.5±14.0 μ*M*, respectively.

Keywords: Phenol; Chlorophenols; 4-(4,5-Diphenyl-1*H*-imidazol-2-yl)benzoyl chloride

1. Introduction

Phenol and chlorophenols are widely used as disinfectants, components of insecticides, herbicides and synthetic fibers. On the other hand, these compounds are considered as pollutants owing to their carcinogenic effects [1]. It has been known that the exposure to benzene or toluene results in an increase of urinary concentration of phenol [2]. From these aspects, the development of a sensitive and selective method for the determination of phenols in biological samples is required.

GC [3] and HPLC [4–7] methods have been exclusively used for determining phenols. In general, the GC method is not suitable for the polar analytes in aqueous samples. In HPLC methods, phenols were determined by spectrophotometry [4] or native

*Corresponding author. Tel.: +81-958-471-111 Ex: 2526; fax: +81-958-423-549; e-mail: naka-ken@net.nagasaki-u.ac.jp

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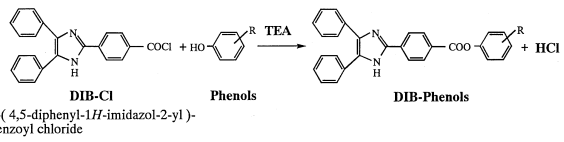

Fig. 1. Reaction scheme of phenols with DIB-Cl.

fluorometry [5]. A few methods with fluorescence [6] or peroxyoxalate chemiluminescence (PO-CL) [7] detections after fluorescent labeling have been reported. The latter labeling methods resulted in an increase in the sensitivity and selectivity in comparison with the non-labeling method.

Recently, we have developed 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl) as a fluorescent labeling reagent for amines and successfully applied it to determine stimulant amines by HPLC [8]. In this study, DIB-Cl was examined as a fluorescence labeling reagent to determine phenolic compounds (i.e., phenol and chlorophenols). DIB-Cl reacts with phenols in the presence of triethylamine (TEA) to produce highly fluorescing derivatives. The reaction scheme of phenols with DIB-Cl is shown in Fig. 1. After optimizing the labeling conditions and HPLC separation conditions of the DIB derivatives, the proposed method was successfully applied to the determination of phenols in human urine.

2. Experimental

2.1. Chemicals

Phenol, 2,4-dichlorophenol and TEA were obtained from Wako (Osaka, Japan). *o*-, *p*-Chlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). DIB-Cl was synthesized according to our previous method [9]. Water was distilled with an Autostill WG 220 (Yamato, Tokyo, Japan) and passed through a Puric-Z (Organo, Tokyo, Japan). Acetonitrile (CH_3CN) and methanol (MeOH) used were of HPLC grade (Wako). The other chemicals used were of analytical reagent grade.

Authentic DIB-phenol was prepared by heating the mixture of DIB-Cl (0.09 g), phenol (0.043 g) and TEA (0.5 ml) in 50 ml CH_3CN at 60~65°C for 1 h. After condensing the mixture, the precipitates obtained were recrystallized from CH_3CN to give white–yellow crystals; yield 0.06 g, m.p. 219~223°C; Anal. Calcd for $C_{28}H_{20}O_2N_2/H_2O$: C, 77.40; H, 5.10; N, 6.45 (%); Found: C, 77.51; H, 5.49; N, 6.98 (%).

2.2. HPLC system

The HPLC system for the determination of phenol derivatives consisted of a CCPD HPLC pump (Tosoh, Tokyo, Japan), a 7125 injector with a 20-μl sample loop (Rheodyne, Cotati, CA, USA), a Daisopak-SP-120-5-ODS-BP (250×4.6 mm I.D., 5 μm, Daiso, Osaka, Japan), an RF-550 fluorescence detector (Shimadzu, Kyoto, Japan) and an FIB-1 recorder (Tosoh). The DIB-phenol derivatives were isocratically separated with CH_3CN–H_2O (76:24, v/v) as an eluent at a flow-rate of 1.0 ml/min and monitored at λ_{ex} 340 and λ_{em} 450 nm.

2.3. Pretreatment of urine sample

The urine samples were obtained from healthy volunteers in our laboratory. For determining total phenols, urine was treated as follows in order to hydrolyze the conjugates [3]: a 100-μl portion of urine was heated with 200 μl of 4 *M* HCl at 60°C for 60 min, cooled to room temperature, and neutralized with 200 μl of 4 *M* NaOH. To 100 μl of urine or hydrolyzed urine, 1.0 ml of 0.1 *M* phosphate buffer (pH 5.5) and 2.5 ml of diethyl ether were successively added. The mixture was vortex-mixed for 1 min and then centrifuged at 900 *g* for 5 min at 4°C. To the organic layer collected (1.5 ml), 10 ml of 3 *M* NaOH were added, vortex-mixed, and evaporated to dryness with a RD-31 centrifugal evaporator (Yamato). The residue was stored at −30°C and dissolved in CH_3CN prior to the analysis. For the determination of free phenols, the hydrolysis step was omitted.

2.4. Derivatization reaction

A 200-μl aliquot of CH_3CN solution of sample or standard phenols was added to 200 μl of 15 m*M* DIB-Cl in CH_3CN containing 1.5 % TEA. The reaction mixture was heated at 60°C for 30 min,

chilled in tap water and to this, 10 μl of 3 *M* HCl and 390 μl of the eluent were added successively. After passing through a membrane filter (0.45 μm), a 20-μl aliquot of the resultant solution was injected into HPLC system.

3. Results and discussion

3.1. Separation conditions

As an eluent, the combinations of CH_3CN–H_2O, MeOH–H_2O and CH_3CN–MeOH were examined. DIB-*m*-chlorophenol and DIB-*p*-chlorophenol could not be separated at all with MeOH–H_2O. Though CH_3CN–MeOH gave an improvement for the separation of DIB-dichlorophenols, DIB-phenol eluted too fast and overlapped with the large blank peaks. On the other hand, by using the mixture of CH_3CN–H_2O (76:24, v/v), five DIB-phenols were isocratically separated within 35 min. The retention times of DIB derivatives of phenol, *o*-chlorophenol, *p*-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol were 12.5, 15.3, 16.9, 23.5 and 34.5 min, respectively. The RSDs of retention times were less than 1.4 % (n=8). A typical chromatogram of DIB-phenols is shown in Fig. 2. The large peak at ca. 28 min in Fig. 2A was derived from DIB-Cl which usually disappeared after the reaction with spiked phenols as shown in Fig. 2B.

Further, we examined the separation conditions so as to include a wide variety of chlorophenolic compounds. The retention times of DIB derivatives of 13 phenols, i.e., phenol, *o*-, *p*-, *m*-chlorophenol, 2,6-, 2,3-, 2,5-, 2,4-, 3,4-, 3,5-dichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and penta-

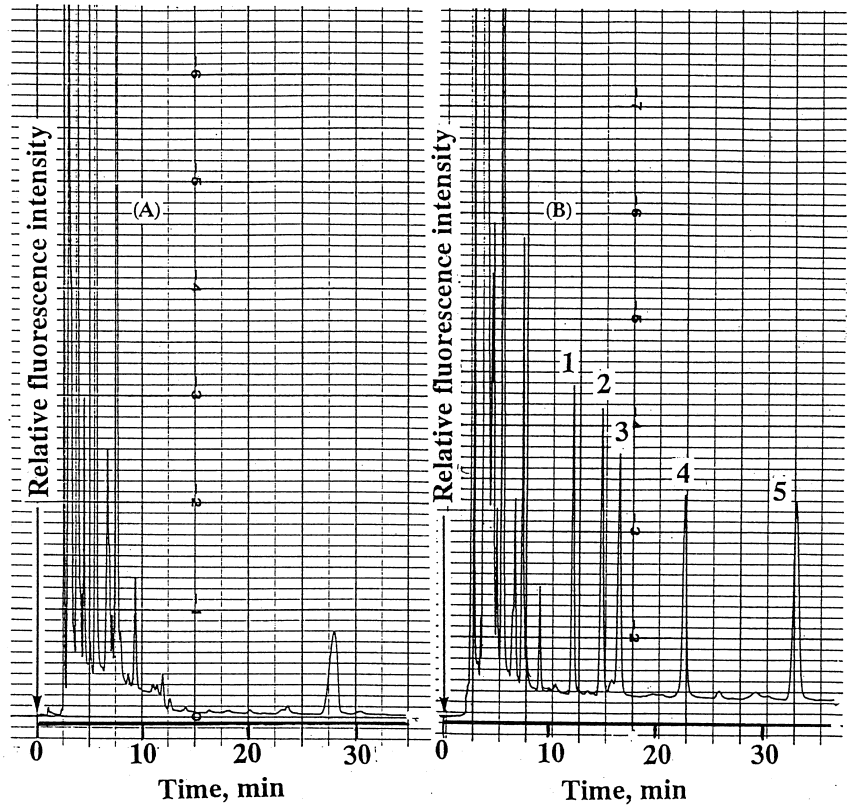

Fig. 2. Chromatogram of reagent blank (A) and DIB derivatives of five standard phenols (B). Peaks: 1=phenol; 2=*o*-chlorophenol; 3=*p*-chlorophenol; 4=2,4-dichlorophenol; 5=2,4,6-trichlorophenol; sample concentrations, 12.5 μ*M* for 1,2,3 and 4; 25.0 μ*M* for 5; HPLC conditions are as in Section 2.

chlorophenol by using $CH_3CN–H_2O–MeOH$ (25:22:53, v/v/v) as an eluent were 24.0, 29.0, 36.5, 37.5, 44.8, 47.5, 51.5, 58.5, 58.5, 70.0, 76.5, 117.8 and 208 min, respectively. Unfortunately, the peaks for DIB derivatives of 2,4- and 3,4-dichlorophenol overlapped and could not be separated from each other. However twelve kinds of DIB-phenols could be separated isocratically. A typical chromatogram of DIB-phenols is shown in Fig. 3

3.2. Reaction conditions

The effect of DIB-Cl concentration on the relative fluorescence intensity (RFI) as a peak height was examined (Fig. 4A). In order to obtain a DIB-Cl solution with high concentration, DIB-Cl was dissolved in CH_3CN containing 1.5% TEA, since it has low solubility in pure CH_3CN. However, as DIB-Cl was unstable in TEA solution, DIB-Cl solutions should be prepared just prior to use. Most of the phenols examined gave the maximum and constant RFI with 10 m*M* of DIB-Cl or more; 15 m*M* DIB-Cl was selected in the following experiments. Prior to the examination of DIB-Cl concentration, TEA and pyridine were tested as basic catalysts. When TEA was used, the peaks of DIB-phenols were sharper and higher than those with pyridine. Thus the effect of TEA concentration was examined in the range of 0.5 to 2.5% (Fig. 4B). The maximum and constant RFIs were obtained with 1.2% of TEA or more; the concentration of TEA was finally chosen to be 1.5% and thus initially added to DIB-Cl CH_3CN solution as described above. Fig. 5 shows the effects of reaction time and temperature on RFI. At both 30°C and 60°C, the maximum and constant RFI was obtained with a reaction time of 20 min or more. No peaks due to the reagent blank were observed to interfere with those of DIB-phenols under the selected conditions of 60°C for 30 min.

The reaction yield of 97.4±4.2% (n=3) was

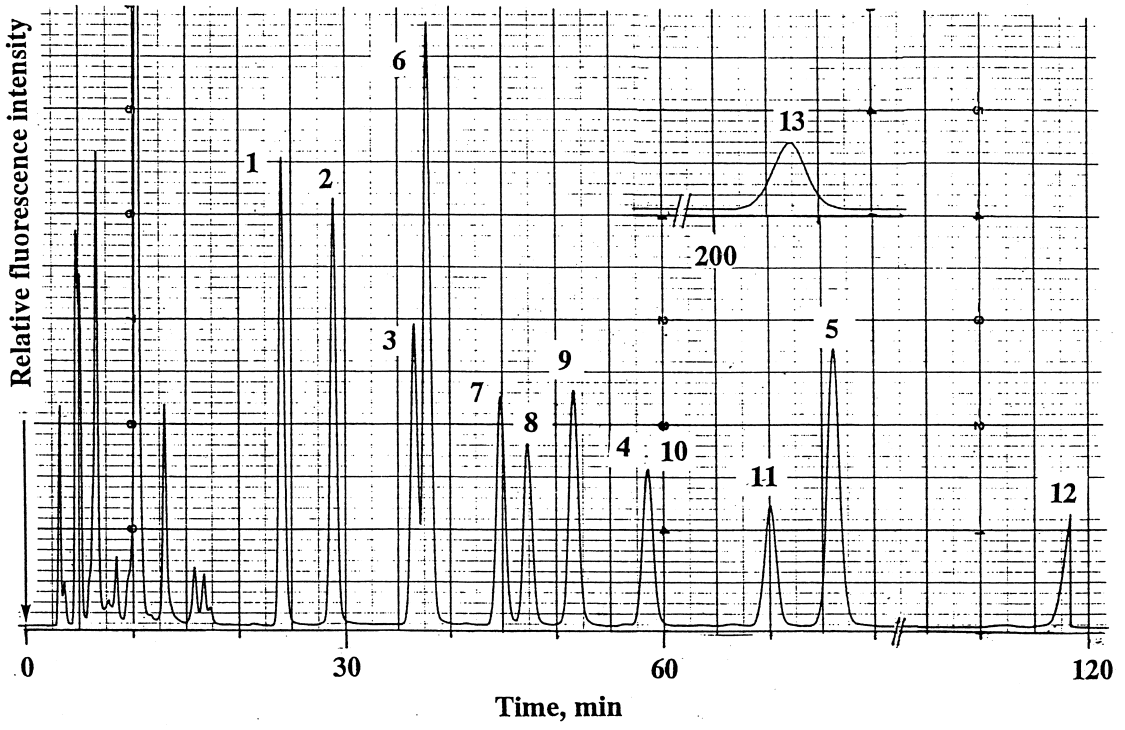

Fig. 3. Chromatogram of DIB-derivatives of 13 standard phenols. Peaks: 1=phenol; 2=*o*-chlorophenol; 3=*p*-chlorophenol; 6=*m*-chlorophenol; 7=2,6-dichlorophenol; 8=2,3-dichlorophenol; 9=2,5-dichlorophenol; 4=2,4-dichlorophenol; 10=3,4-dichlorophenol; 11=3,5-dichlorophenol; 5=2,4,6-trichlorophenol; 12=2,3,4,6-tetrachlorophenol and 13=pentachlorophenol; sample concentration, 125 μ*M*; eluent, $CH_3CN–H_2O–MeOH$(25:22:53, v/v/v).

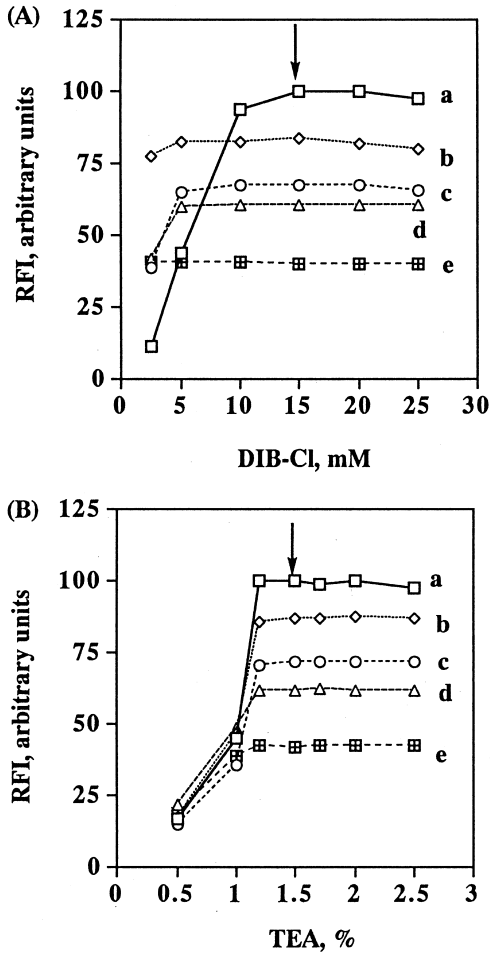

Fig. 4. Effects of DIB-Cl (A) and TEA (B) concentrations on RFI. Sample: 20 μ*M* phenols; curves: a=phenol; b=*o*-chlorophenol; c=*p*-chlorophenol; d=2,4-dichlorophenol; e=2,4,6-trichlorophenol. HPLC conditions are as in Section 2.

estimated by comparing the peak heights of authentic DIB-phenol and the reaction product.

3.3. Calibration curves and detection limits

Calibration curves prepared with standard phenols showed good linearities at concentrations of phenols ranging from 0.4 to 100.0 μ*M* (r=1.000). The detection limits for phenol, *o*-, *p*-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol at a S/N of 3 were 0.08 (0.4), 0.024 (0.12), 0.04 (0.2), 0.033 (0.17) and 0.05 μ*M* (0.23 pmol/20 μl injection), respectively. The sensitivity of the proposed method

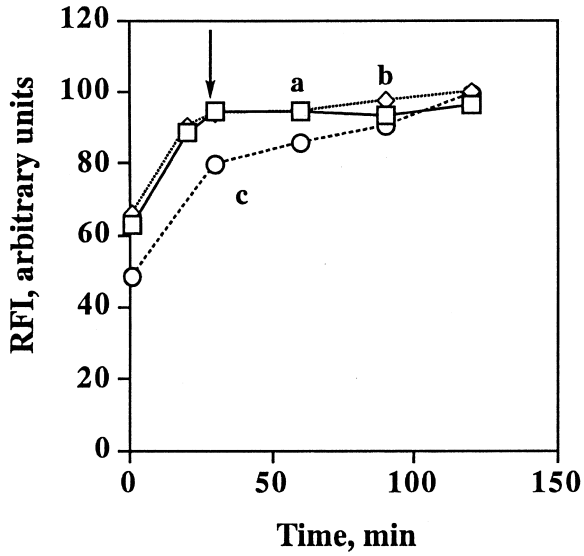

Fig. 5. Effects of reaction time and temperature on RFI. Sample: 20 μ*M* phenol; curves: a=30°C; b=60°C; c=90°C. HPLC conditions are as in Section 2.

was almost ten times higher than those of GC [3] and HPLC with native fluorometric detection [4], and comparable to that of HPLC with labeling technique [6].

The RSDs of the proposed method for ten replicate measurements ranged from 1.5 to 5.5% at the concentration of 40 μ*M* standard phenols.

3.4. Stability of DIB phenol derivatives

The stability of DIB-phenols after the labeling reaction was evaluated (Fig. 6). The reaction mixture in a vial was stored in the dark at room temperature and 20-μl aliquots were injected at specific intervals over 24 h. DIB derivatives were so stable that no decrease of RFI was observed at least for 24 h, which is advantageous for preparing and analyzing many samples.

3.5. Accuracy and reproducibility

The recoveries of DIB-phenols from spiked urine and within-day precisions were determined using urine samples containing 7.5 μ*M* and 75 μ*M* of

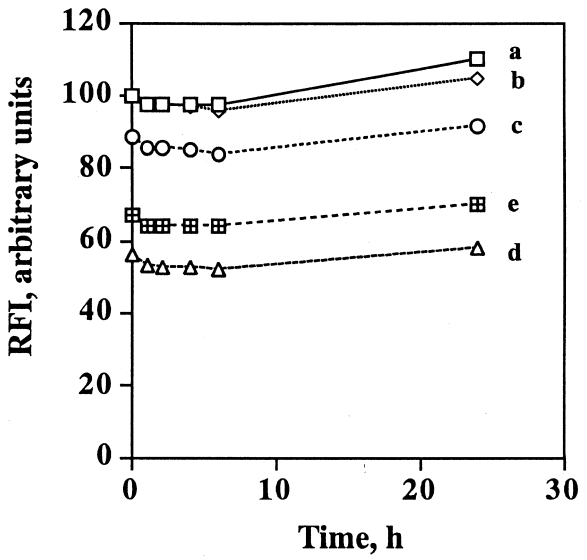

Fig. 6. Stability of DIB-phenol derivative in the dark at room temperature. Sample: 20 μ*M* phenols. HPLC conditions are as in Section 2.

phenols for the measurement of free and total phenols, respectively. The recoveries and RSDs obtained were 78.8–92.3% and 1.6–7.0% for free phenols, and 88.5–100.7% and 2.3–4.3% for total phenols ($n=5$), respectively.

The between-day precisions were also determined by analyzing the same samples over a period of one week ($n=3$). RSDs obtained for free and total phenols were in the range of 4.0 to 13.8% and 8.3 to 14.2%, respectively. The results are summarized in Table 1.

3.6. Determination of phenols in urine

The average concentrations of free and total phenol determined in six urine samples were 4.3±2.5 and 29.5±14.0 μ*M* ($n=6$), respectively. It is known that phenol normally occurs in the human body as a metabolite of tyrosine by intestinal flora [2]. The concentrations obtained with the proposed method were within the concentration range reported by Murray et al. (5.6–184 μ*M*) [10]. It has also been reported that 2,4,6-trichlorophenol was detected in urine from sawmill workers [2], and *p*-chlorophenol was detected in urine from subjects exposed to monochlorobenzene [11]. However, chlorophenols could not be determined in any sample we have investigated. This means the volunteers in our laboratory have not been regularly exposed to chlorophenols. Typical chromatograms obtained with a hydrolyzed urine sample and one spiked with phenols are shown in Fig. 7. Chromatograms for a

Table 1
Precision and accuracy of the proposed method for phenols in hydrolyzed urine

Compound	Added (μ*M*)	Within-day ($n=5$)			Between-day ($n=3$)	
		Found±SD (μ*M*)	RSD (%)	Accuracy (recovery, %)	Found±SD (μ*M*)	RSD (%)
Phenol	0	30.6[a]	–	–	31.1±3.2	10.3
	75.0	106.1±4.6	4.3	100.7	117.6±13.6	11.6
o-Chlorophenol	0	n.d.[b]	–	–	n.d.[b]	–
	75.0	71.1±2.3	3.2	94.8	78.3±6.5	8.3
p-Chlorophenol	0	n.d.[b]	–	–	n.d.[b]	–
	75.0	66.4±1.8	2.7	88.5	75.0±7.2	9.6
2,4-Dichlorophenol	0	nd.[b]	–	–	n.d.[b]	–
	75.0	72.5±2.5	3.4	96.7	80.0±10.0	12.5
2,4,6-Trichlorophenol	0	n.d.[b]	–	–	n.d.[b]	–
	75.0	74.2±1.7	2.3	98.9	58.3±8.3	14.2

[a] Mean of duplicate measurements.
[b] n.d.=not detected.

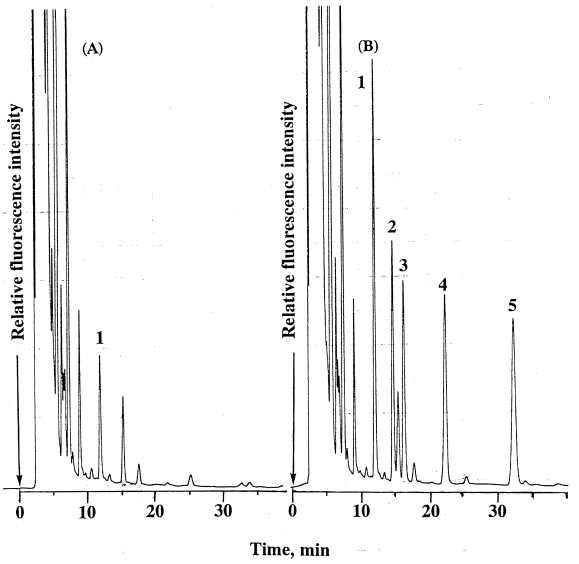

Fig. 7. Chromatograms of a hydrolyzed urine (A) and urine spiked with standard phenols (B). Sample: hydrolyzed urine (A) and urine spiked with 125 μ*M* of phenol, *o*- and, *p*-chlorophenols and 2,4-dichlorophenol, and 250 μ*M* of 2,4,6-trichlorophenol. HPLC conditions are as in Section 2.

normal urine sample and one spiked with phenols are also shown in Fig. 8.

4. Conclusions

In this paper, we developed a sensitive HPLC method for the determination of phenol and chlorophenols and applied it to the assay of human urine samples. Separation of five or twelve kinds of representative DIB-phenol derivatives were achieved isocratically in 35 or 208 min, respectively. The DIB derivatives were found to be stable at least for 24 h when they were kept at room temperature in the dark. The sensitivity of the proposed method was ten times higher than GC [3], and HPLC methods employing spectrophotometric [4] or native fluorometric detections [5]. The HPLC system of the proposed method was more simple compared to that of the PO-CL method [7]. Many more kinds of chlorophenols could be simultaneously determined by the proposed method than by the fluorometric

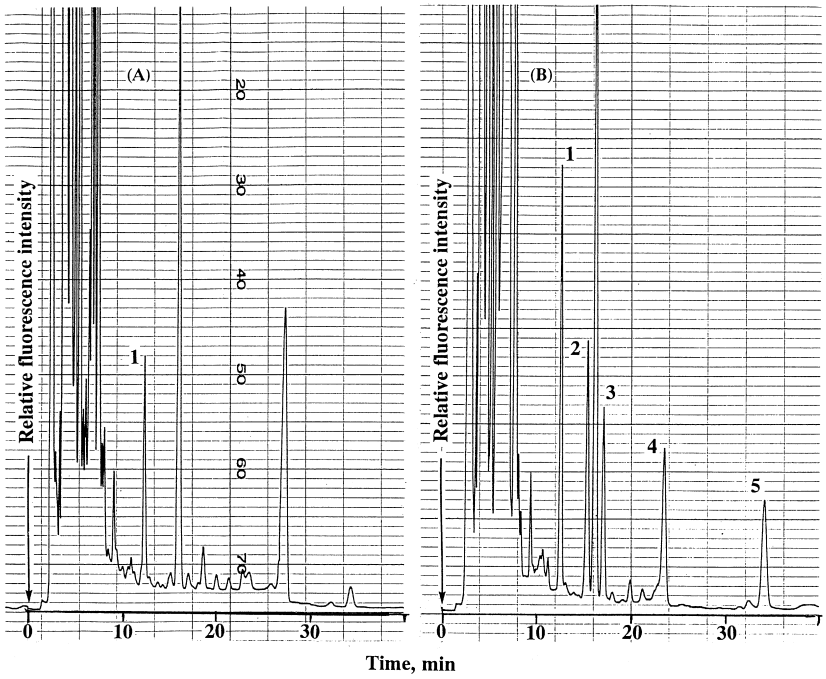

Fig. 8. Chromatograms of normal urine (A) and urine spiked with standard phenols (B). Sample: normal urine (A) and urine spiked with 7.5 μ*M* of phenol, *o*-chlorophenol, *p*-chlorophenol and 2,4-dichlorophenols, and 15 μ*M* of 2,4,6-trichlorophenol. HPLC conditions are as in Section 2.

method [6]. We could demonstrate that the present method is applicable to determine phenols in urine and concluded that it is useful for biological monitoring of phenols in environmental and toxicological investigations.

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