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NEW DERIVATIZATION OF AMINES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

N-(4-Anilinophenyl)isomaleimide (APIM) and N-(4-anilinophenyl)isophthalimide (APIP) were prepared and evaluated for precolumn derivatization of amines in liquid chromatography with electrochemical detection. Phenethylamine and piperidine were taken as the model compounds for primary and secondary amines, respectively. Derivatization of amines with these reagents was complete within 20 min at room temperature in acetonitrile–0.05 *M* borate buffer (pH 9.0) (1:1). The derivatives formed with APIM were more responsive to an electrochemical detector than those formed with APIP. The detection limit for the phenethylamine–APIM adduct was *ca.* 0.1 pmol. The electrochemical response was found to be linear in the range 0.1–10 ng of phenethylamine.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a useful tool for the separation and determination of various compounds with a wide range of polarities and molecular weights. Among several types of detector hitherto developed, ultraviolet (UV) and fluorescence detectors are most frequently employed. Various derivatization reagents are used for UV or fluorescence detection[1,2].

The electrochemical detector has been shown to be sensitive and selective for the analysis of electroactive compounds in a complex matrix[3]. The derivatization reagents of potential utility for HPLC with electrochemical detection (ED) have been reviewed[4]. As for alkylamines, Caudill and co-workers[5,6] and Jacobs and Kissinger[7] proposed the use of 2,4,6-trinitrobenzenesulphonic acid as a derivatization reagent for reductive ED. This reagent is so sensitive that a picomole level of amino acids can be determined. In reductive ED, however, the presence of oxygen exerts significant interferences, such as a large baseline offset and increased faradaic noise. To overcome these problems, tedious and time-consuming deoxygenation of the mobile phase and sample is required prior to the analysis. On the contrary, oxidative ED is unaffected by dissolved oxygen and is as sensitive as the reductive mode. Therefore, we have previously proposed N-succinimidyl homovanillate (SIHV) as a useful derivatization reagent for amines in oxidative ED[8].

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The development of more sensitive and selective derivatization reagents, which could permit oxidative ED of a picomole level of amines, has been undertaken. The present paper deals with the preparation of N-(4-anilinophenyl)isomaleimide (APIM) and N-(4-anilinophenyl)isophthalimide (APIP) and their use for precolumn derivatization in HPLC–ED. The structures are shown in Fig. 1.

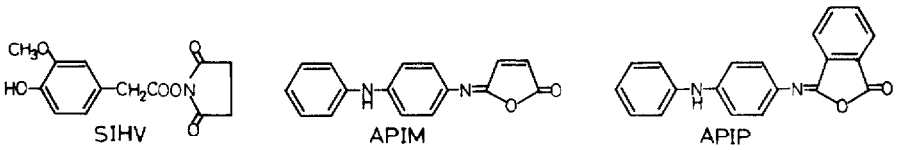

Fig. 1. Structures of derivatization reagents.

EXPERIMENTAL

Instruments

The apparatus used was a Waters Model ALC/GPC-202 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Yanagimoto Model VMD-101 electrochemical detector with a glassy carbon electrode (Yanagimoto, Kyoto, Japan). The sample was introduced by a Waters Model U6K sample loop injector with an effective volume of 2 ml. A Waters μBondapak C_{18} (5 μm) column (30 × 0.39 cm I.D.) was used at ambient temperature. A mixture of acetonitrile and 0.5% (w/v) ammonium dihydrogen phosphate (pH 4.5) was used as mobile phase at a flow-rate of 1.0 ml/min. Mass spectra were recorded on a Hitachi Model M-52 spectrometer. Nuclear magnetic resonance spectra were obtained on a JEOL Model FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard (abbreviations used: s = singlet; d = doublet; m = multiplet).

Chemicals and reagents

All the reagents were purchased from Tokyo Kasei (Tokyo, Japan). Solvents were purified by distillation prior to use. Silica gel HF_{254} (Merck, Darmstadt, F.R.G.) was used for thin-layer chromatography (TLC). N-(4-Anilinophenyl)maleamic acid and SIHV were prepared in these laboratories by the methods previously reported[8,9].

Derivatization of amines

The reaction was carried out at room temperature in acetonitrile–0.05 *M* borate buffer (pH 9.0) (1:1) (0.2 ml) and terminated by addition of 2% methylamine hydrochloride solution (0.1 ml). An aliquot of the solution was applied to HPLC. The derivatization reagents dissolved in acetonitrile could be stored unchanged in a refrigerator at least for one month.

Preparation of derivatization reagents

N-(4-Anilinophenyl)isomaleimide (APIM). N-(4-Anilinophenyl)maleamic acid (500 mg) and sodium acetate (250 mg) were added to acetic anhydride (4 ml), and the whole was heated at 90–100°C for 5–10 min until the solution became transparent and then was poured into ice water. The brown precipitate was collected by

filtration and recrystallized from ethyl acetate to give APIM (300 mg) as reddish brown needles [m.p. 158–159°C; IR ν_{max}^{KBr} (cm^{-1}): 3340 (–NH–); 1750 (=C=O); 1655 (=C=N–); ^1H NMR (C^2HCl_3)δ: 5.91 (1H, broad s, –NH–), 6.55 (1H, d, J=5.7 Hz, vinyl H), 7.34 (1H, d, J=5.7 Hz, vinyl H), 6.96–7.56 (9H, m, aromatic H); analysis calculated for $C_{16}H_{12}N_2O_2$: C, 72.71; H, 4.58; N, 10.60; found: C, 72.45; H, 4.57; N, 10.46. MS m/z: 264 (M^+)].

N-(4-Anilinophenyl)isophthalimide (APIP). A solution of phthalic anhydride (1.92 g) in dichloromethane (5 ml) was added dropwise to 4-aminodiphenylamine (2.0 g) in dichloromethane (10 ml) under ice-cooling. After being allowed to stand at room temperature overnight, the resulting precipitate was collected by filtration, washed with dichloromethane, and dried to give N-(4-anilinophenyl)phthalamic acid (2.0 g) as a gray powder [m.p. 268–270°C (decomp.)]. The crude product was subjected to further treatment without purification.

A solution of N,N'-dicyclohexylcarbodi imide (310 mg) in dichloromethane (2 ml) was added dropwise to a stirred suspension of N-(4-anilinophenyl)phthalamic acid (500 mg) in dichloromethane (5 ml), and the whole was allowed to stand at room temperature for 2 h. After removal of the resulting dicyclohexylurea by filtration, the filtrate was evaporated down. The residue was purified by column chromatography on silica gel using ethyl acetate–*n*-hexane as eluent. Recrystallization of the eluate from ethyl acetate–*n*-hexane gave APIP (200 mg) as yellow needles [m.p. 120–121°C; IR ν_{max}^{KBr} (cm^{-1}): 3350 (–NH–); 1770 (=C=O); 1684 (=C=N–); ^1H NMR (C^2HCl_3)δ: 5.87 (1H, s, –NH–), 6.88–8.06 (13H, m, aromatic H); analysis calculated for $C_{20}H_{14}N_2O_2$: C, 76.42; H, 4.49; N, 8.91; found: C, 76.23; H, 4.65; N, 9.11; MS m/z: 314 (M^+)].

Preparation of APIM adducts

Phenethylamine. APIM (50 mg) was added to a solution of phenethylamine hydrochloride (50 mg) in dichloromethane (10 ml)–triethylamine (1.5 ml), and the whole was kept at room temperature for 1 h and then evaporated down. The yellow oily residue was subjected to preparative TLC using *n*-hexane–ethyl acetate (1:1) as a developing solvent. Elution of the adsorbent corresponding to the spot (R_F = 0.17) with ethyl acetate gave the adduct (60 mg) as yellow needles [m.p. 138–139°C; ^1H NMR (C^2HCl_3) δ: 2.87 (2H, t, J=5.7 Hz, $-CH_2-C_6H_5$); 3.61 (2H, m, $-CONHCH_2CH_2-$); 5.96 (1H, d, J_{AB}=14 Hz, vinyl H); 6.16 (1H, d, J_{AB}=14 Hz, vinyl H), 6.88–7.64 (14H, m, aromatic H); MS m/z: 385 (M^+)].

Piperidine. Piperidine (two drops) was added to a solution of APIM (30 mg) in dichloromethane (1 ml), and the whole was kept at room temperature for 1 h and then evaporated down. The yellow oily residue was subjected to preparative TLC using ethyl acetate–*n*-hexane (2:1) as a developing solvent. Elution of the adsorbent corresponding to the spot (R_F = 0.19) with ethyl acetate gave the adduct (20 mg) as yellow needles [m.p. 149–150°C; ^1H NMR (C^2HCl_3)δ: 1.80 (6H, broad s, $-(CH_2)_3-$); 3.52 (4H, m, $-CH_2\underset{|}{N}CH_2-$); 6.20 (1H, d, J_{AB} = 13.7 Hz, vinyl H); 6.42 (1H, d, J_{AB} = 13.7 Hz, vinyl H); 6.80–7.56 (9H, m, aromatic H); MS m/z: 349 (M^+)].

Preparation of APIP adducts

Phenethylamine. APIP (66 mg) was added to a solution of phenethylamine hydrochloride (100 mg) in dichloromethane (2 ml)–triethylamine (1 ml), and the

whole was kept at room temperature overnight and then evaporated down. The oily residue was purified by preparative TLC using ethyl acetate–*n*-hexane (1:1) as a developing solvent. Elution of the adsorbent corresponding to the spot (R_F = 0.47) with ethyl acetate gave the adduct (20 mg) as a colourless amorphous substance [m.p. 198–199°C; ^{1}H NMR (C^2HCl_3)δ: 2.86 (2H, t, J=7.1 Hz, CH_2–C_6H_5); 3.68 (2H, m, –CONHCH_2CH$_2$–); 6.88–7.86 (18H, m, aromatic H); MS m/z: 435 (M^+)].

Piperidine. Piperidine (one drop) was added to a solution of APIP (10 mg) in dichloromethane (1 ml), and the whole was kept at room temperature for 1 h and then evaporated down to give the adduct (12 mg) as a colourless amorphous substance [m.p. 272–273°C; ^{1}H NMR (C^2HCl_3)δ: 1.54 (6H, broad s, –$(CH_2)_3$–); 3.13 (4H, m, –CH_2NCH_2–); 6.80–7.96 (13H, m, aromatic H); MS m/z: 399 (M^+)].

Preparation of the amide from phenethylamine and SIHV

N,N′-Dicyclohexylcarbodiimide (150 mg) was added to a solution of homovanillic acid (70 mg) and phenethylamine hydrochloride (100 mg) in pyridine (3 ml), and the whole was allowed to stand at room temperature overnight and then evaporated down. The oily residue was subjected to preparative TLC using ethyl acetate–*n*-hexane (1:1) as a developing solvent. Elution of the adsorbent corresponding to the spot (R_F = 0.27) with ethyl acetate gave the amide (100 mg) as a colourless oily substance [^{1}H NMR (C^2HCl_3)δ: 2.71 (2H, t, J=5.7 Hz, –CH_2–C_6C_5); 3.43 (4H, m, –CONHCH_2CH$_2$– and –CH_2CO–), 3.80 (3H, s, –OCH_3), 6.56–7.24 (8H, m, aromatic H); MS m/z: 285 (M^+)].

RESULTS AND DISCUSSION

We have previously reported the development of N-(anilinophenyl)maleimide (APM) as a sensitive derivatization reagent for thiol compounds[9]. The design of a promising derivatization reagent for amines requires similarly the incorporation of suitable structural features: functions reactive toward the amino group and highly responsive to an electrochemical detector. It is well substantiated that the N-substituted isomaleimide and isophthalimide readily condense with primary and secondary amines to form peptide bonds[10,11]. Accordingly, we prepared APIM and APIP for use in derivatization of amino compounds.

APIM was prepared from the corresponding malcamic acid by brief treatment with acetic anhydride in the presence of sodium acetate at 90–100°C. The reaction was terminated immediately after the reaction mixture became transparent. Otherwise the prolonged reaction caused the isomerization of APIM to APM[12].

An initial attempt to prepare APIP in similar fashion resulted in failure. Accordingly, APIP was synthesized from the corresponding phthalamic acid by treatment with N,N′-dicyclohexylcarbodiimide in dichloromethane[12]. Both APIM and APIP proved to be stable at least for one year, when kept dry and in the dark at room temperature.

The reaction of phenethylamine with each derivatization reagent was carried out in organic solvent–borate buffer (pH 9.0) (1:1) at room temperature for 1 h (Table I). As for both APIM and APIP, the use of acetonitrile, acetone and methanol gave decreasing electrochemical response in that order. In the case of SIHV, however, no significant solvent effect on the reaction rate was observed. Therefore, acetonitrile was chosen as a suitable organic solvent for the derivatization reaction.

TABLE I

EFFECT OF ORGANIC SOLVENTS ON THE REACTION RATE

The reaction was carried out at room temperature in organic solvent–0.05 *M* borate buffer (pH 9.0) (1:1).

Reagent	*Reaction rate (%)*			
	Acetonitrile	*Acetone*	*Methanol*	*Ethanol*
APIM	100	77.3	10.6	19.7
APIP	96.5	79.2	23.0	–
SIHV	100	100	102	–

The reactivities of the three derivatization reagents toward the amino group were tested for phenethylamine as a model compound. A solution of phenethylamine hydrochloride (1.0 μg) and the derivatization reagent (60 equivalents) in acetonitrile–0.05 *M* borate buffer (pH 9.0) (0.2 ml) was allowed to stand at room temperature. The reaction was terminated by addition of 2% methylamine hydrochloride solution (0.1 ml) at 5, 10, 20, 30, 60, 90 and 120 min after the initiation. The time courses of derivatization of phenethylamine with the three reagents were investigated. The reaction rate increased along with the reaction time up to 20 min then reached a plateau. Both APIM and APIP reacted with phenethylamine almost quantitatively, but the conversion rate of the latter was somewhat lower than that of the former. These two exhibited similar reactivities toward the secondary amino group of piperidine, yielding the derivatives quantitatively in 20 min. The retention values of these derivatives in HPLC are collected in Table II.

The sensitivity of an electrochemical detector to each derivative was estimated at various potentials. The peak area of the derivatized phenethylamine was measured at different potentials and compared with that obtained with the phenethylamine–APIM adduct at +0.60 V *vs.* an Ag/AgCl reference electrode (Fig. 2). Of the three derivatives tested, the APIM derivative produced the best response from the detector and most readily oxidized. When the applied potential was set at +0.60 V, the detector was 1.2 and 4.2 times more sensitive to the APIM adduct than to the APIP and SIHV derivatives, respectively. It is to be noted that the diphenylamino group

TABLE II

RETENTION VALUES OF THE ADDUCTS FORMED FROM AMINES WITH DERIVATIZATION REAGENTS

*Derivative**	*Mobile phase acetonitrile–buffer*	t_R *(min)*	t_O *(min)*	*k'*
APIM–Phe	3:2	6.60	1.80	2.67
APIP–Phe	5:3	6.20	1.93	2.22
SIHV–Phe	3:5	8.10	2.38	2.40
APIM–Pip	1:1	7.98	1.92	3.16
APIP–Pip	3:2	6.90	1.98	2.48

* Phe = phenethylamine, Pip = piperidine.

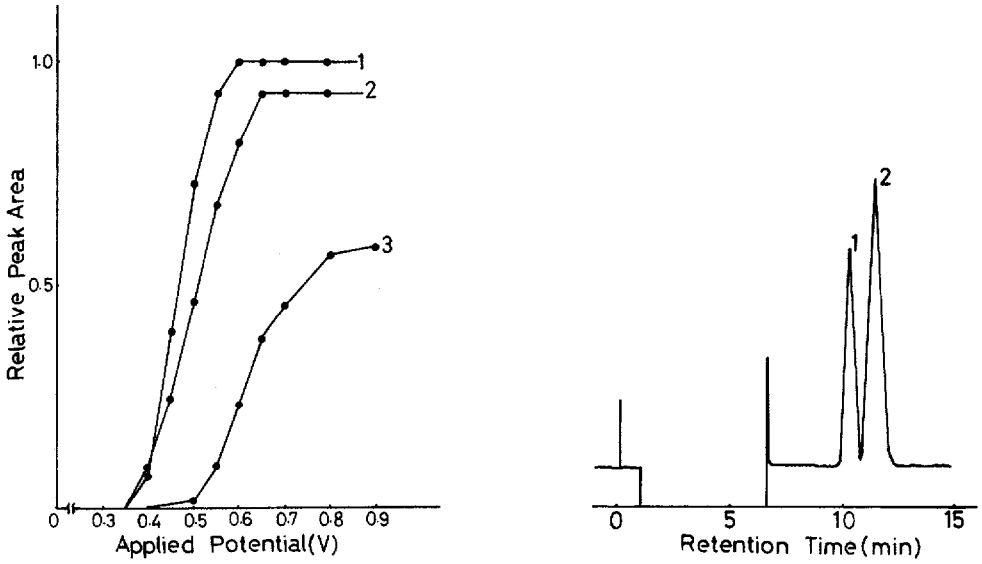

Fig. 2. Responses of phenethylamine derivatives formed with APIM (1), APIP (2) and SIHV (3) at various applied potentials.

Fig. 3. A chromatogram of tryptamine (1) and phenethylamine (2) (each 5 ng) derivatized with APIM.

would be more favourable for ED than the guaiacol group. The detection limit of the phenethylamine–APIM adduct was 13 pg at 4 nA full scale (signal-to-noise ratio = 2). It is concluded that APIM is the most suitable derivatization reagent with respect to sensitivity and selectivity. On the chromatogram the APIM adduct was completely separated from the peaks due to excess reagent and other ghost peaks. A linear correlation was observed between the peak height and injected amount over a range from 0.1 to 10 ng of phenethylamine.

Phenethylamine and tryptamine were derivatized with APIM and were then subjected to HPLC. They produced sharp peaks of the theoretical shape which were satisfactorily separated from each other and detected with high sensitivity (Fig. 3). The connector between the column and the detector was removed for *ca.* 5 min to protect the glassy carbon working electrode from the excess of derivatization reagent. Both phenethylamine and tryptamine are excreted in urine of patients[13,14] as well as healthy subjects[15]. The use of APIM may serve to develop a sensitive method for the quantitation of these arylalkylamines in biological fluids by HPLC–ED.

CONCLUSION

Of the three reagents studied APIM proved to be most suitable for precolumn derivatization in HPLC–ED. It can be readily prepared from commercially available starting materials and is stable in the crystalline state at least for one year when kept at room temperature. The derivatization reaction proceeds readily to provide the adduct, which has favourable electrochemical properties. Further studies on the application of the proposed method to biological specimens are being conducted in these laboratories.

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