NEW LABELING AGENT FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ALCOHOLS WITH FLUORESCENCE AND ELECTROCHEMICAL DETECTION

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Abstract New labeling agent, 2-[2-(azidocarbonyl)ethyl]-3-methyl-1,4-naphthoquinone (AMQ), has been synthesized and developed for the analysis of alcohols in HPLC with fluorescence or electrochemical detection after post column reduction with platinum catalyst. Optimal reaction conditions of AMQ were examined with 1-eicosanol selected as a model compound of alcohols. Alcohols were reacted with the reagent in acetone at 100°C for 15 min to form the corresponding carbamic acid esters, which were separated on an Inertsil C8 reversed-phase column with aqueous methanol containing 0.05M sodium perchlorate. The detection limit for 1-eicosanol with electrochemical detection was 16 fmol/10µl injection volume.

Key words 2-[2-(azidocarbonyl)ethyl]-3-methyl-1,4-naphthoquinone (AMQ), HPLC, fluorescence, electrochemical detection, platinum catalyst

Various UV or fluorescence derivatization reagents have been reported for the determination of alcohols by high performance liquid chromatography (HPLC) [1-6]. In contrast, only a few reagents for electrochemical detection (ED) have been developed for the derivatization of alcohols [7]. In the present work, new labeling agent, 2-[2-(azidocarbonyl)ethyl]-3-methyl-1,4-naphthoquinone (AMQ), has been synthesized and investigated its reactivity with alcohols, carboxylic acids and amines etc. Furthermore, this new agent has been developed for the analysis of alcohols in HPLC with fluorescence or ED after post column reduction with platinum catalyst.

EXPERIMENTAL

Reagents Alcohols and 2-methyl-1,4-naphthoquinone were purchased from Tokyo Kasei Co. Ltd.(Tokyo, Japan). Sodium azide was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All other chemicals and solvents employed were of analytical reagent grade. Organic solvents were distilled and dried in the usual manner. 2-Carboxyethyl-3-methyl-1,4-naphthoquinone (AMQ-COOH) was prepared according to the method by Okamoto et al.[8].

Apparatus HPLC system used in this work was a Hitachi L-6200 pump equipped with a detector of Hitachi L-4200 UV-VIS spectrophotometer (280nm), Shimadzu RF-530 fluorescence spectrometer (Ex 320nm, Em 430nm) or TOA ICA 3060 amperometric detector with glassy carbon working electrode and a Ag/AgCl reference electrode (applied voltage +0.7V), and with an integrator of Shimadzu C-R6A. An Inertsil C8 column (15cm x 4.0mm I.D., particle size 5µm; GL Sciences, Tokyo, Japan) was used at ambient temperature. The mobile phase for the separation of the AMQ derivatives of the examined alcohols was 90% (v/v) methanol containing 0.05M sodium perchlorate at a flow rate of 1.0ml/min. The effluent from the column was directly passed through the platinum catalyst column (10 x 4.6mm I.D. in stainless-steel column). This column was packed with platinum catalyst (5% on alumina, 10µm, provided by Toa Electronics Ltd., Tokyo, Japan) by tapping, and purged with water at a flow rate of 10ml/min for 5min.

Synthesis of AMQ To AMQ-COOH (3.35g, 13mmol) dissolved in 10ml of dry benzene, oxalyl chloride (2g, 16mmol; Wako, Osaka, Japan) was added. The mixture was stirred at 60°C for 15min, and then the reaction mixture was evaporated to dryness *in vacuo*. To the residue dissolved in 10ml of dry acetone, activated sodium azide (1.3g, 20mmol) was added. The mixture was stirred at 0°C for 2h, then the reaction mixture was evaporated to dryness *in vacuo*, and the residue was dissolved in ethyl acetate and purified by column chromatography on silica gel with ethyl acetate-n-hexane (1:10). AMQ (2.55g, 8.6mmol, 65%). mp 56.5-58°C. ¹H-NMR (CDCl₃) δ: 2.2 (3H, s), 2.4-2.7 (2H, m), 2.8-3.2 (2H, m), 7.5-8.2 (4H, m). IR (CH₂Cl₂) cm⁻¹: 1662, 1725, 2150. Ms m/e: 269

Chemical analysis of AMQ-benzyl alcohol derivative AMQ (200mg, 0.7mmol) and benzyl alcohol (70mg, 0.6mmol) were dissolved in 2ml of acetone. The solution was placed in a test tube (10ml), heated at 100°C for 30min and then cooled to room temperature. The reaction mixture was then evaporated to dryness *in vacuo*, and the residue was dissolved in 2ml of ethyl acetate and chromatographed on a silica gel C-200 (Wako, Osaka, Japan) with n-hexane-ethyl acetate (5: 1). The main fraction was evaporated to dryness under reduced pressure, and the residue was recrystallized from n-hexane-ethyl acetate to give AMQ-benzyl alcohol derivative as yellow needle (135mg, 60%). mp 101-103°C. $^1\text{H-NMR}$ (CDCl_3) δ : 1.7 (1H, s), 2.2 (3H, s), 2.8-3.1 (2H, m), 3.2-3.6 (2H, m), 5.1 (2H, s), 7.3 (5H, s), 7.5-8.2 (4H, m). IR (CH_2Cl_2) cm^{-1} : 1662, 1725, 3450. Ms m/e : 349.

Derivatization procedure for HPLC analysis Five microliter of a test solution of alcohols in benzene (2.5 $\mu\text{l}/\text{ml}$, each) was placed in a test tube (10ml), and then 200 μl of 0.1% AMQ in acetone was added. The mixture was heated at 100°C for 15min. After cooling, 1ml of methanol was added to the mixture, and 10 μl of the reaction mixture was injected into the chromatograph.

RESULTS AND DISCUSSION

AMQ was readily prepared from 2-carboxyethyl- 3-methyl-1,4-naphthoquinone. This structure was confirmed by the $^1\text{H-NMR}$, IR and mass spectral data (Fig. 1). In order to investigate the reactivity of

Fig. 1 Synthesis of AMQ and its reaction with alcohols

AMQ with alcohols, we have used 1-eicosanol as a model compound. The labeling yield was evaluated from the ratio of the HPLC-UV response of the labeling preparation vs. that of an authentic sample. In this study, we found acetone was a suitable labeling solvent in the reactivity compared with the other solvents, ethyl acetate, dichloromethane, acetonitrile or benzene. The derivatization reaction with 1-eicosanol proceeded rapidly with increasing reaction temperature. The reaction was

Fig. 2 Effects of the reaction time and temperature on AMQ derivatization of 1-eicosanol

completed with heating at 100°C for 15 min (Fig. 2). AMQ gave the intense peak height at the concentrations more than 0.05%, so enough volumes of 0.1% reagent solution were chosen for the

Fig. 3 Chromatograms of AMQ derivatives of alcohols

A 5 μ l portion of alcohols solution (2.5 μ mol/ml, each) was treated with AMQ as described in Experimental.

A: UV. B: Fluorescence. C: ED. Peaks 1: 1-Tetradecanol 2: 1-Pentadecanol

3: 1-Hexadecanol 4: 1-Heptadecanol 5: 1-Stearyl alcohol 6: 1-Eicosanol

procedure of the derivatization. Constant peak heights were obtained by the AMQ concentration of 0.1% in the reaction solution at 100°C for 15min. The conversion efficiency of the alcohols to the

Table 1. Retention times and detection limits of AMQ derivatives of alcohols for ED

Compound	Mobile phase Aqueous methanol (%, v/v)	Retention time (min)	Detection limit ^{a)} (fmol)
n-Butanol	70	6.7	16
i-Butanol	70	6.4	17
sec-Butanol	70	6.1	15
t-Butanol	70	6.0	23
Benzyl alcohol	70	6.2	14
Cyclohexyl alcohol	70	8.7	15
1-Decanol	90	4.2	9
1-Undecanol	90	4.7	11
1-Dodecanol	90	5.9	8
1-Tridecanol	90	6.3	8
1-Tetradecanol	90	7.6	12
2-Tetradecanol	90	7.1	10
1-Pentadecanol	90	8.8	9
1-Hexadecanol	90	10.4	8
1-Heptadecanol	90	12.3	9
1-Stearyl alcohol	90	14.6	11
1-Eicosanol	90	21.2	16

a) The amount in the injected volume (10 μ l) giving a S/N of 3.

AMQ derivatives was examined by comparing the peak heights of the labeling preparations with those of the pure reaction products; the conversion rates were 96% (1-eicosanol), 45%

(2-tetradecanol) and 10% (estrone), respectively. As the optimization conditions for HPLC-ED, the ED operating at +0.7V as the applied potentials, and the aqueous MeOH containing 0.05M sodium perchlorate as a mobile phase gave the maximal response for the determination of alcohols. Figure 3 shows the typical chromatogram of the various alcohols derivatized with AMQ. The resulting adduct could be detected by UV, fluorescence and ED with a detection limit of 1.6pmol, 0.1pmol and 16fmol for 1-eicosanol, respectively, at a signal-to-noise ratio of 3. The AMQ derivatives were stable at least for 4 days in the dark at 0°C. The retention times and detection limits for the AMQ derivatives of the alcohols were summarized in Table 1. Other substances such as carboxylic acids, aldehydes, ketones and amino acids were not reacted with AMQ. Although hydroxycarboxylic acids were reacted with AMQ, no definitive peaks on the chromatogram were observed under the HPLC conditions employed. Amines (aniline, benzyl-, phenethyl- and cyclohexyl-amine) reacted with AMQ at room temperature, however, the products were not urea derivatives but amides. This newly developed derivatization reagent for alcohols in HPLC with fluorescence or ED was proved to be satisfactory with respect to sensitivity and reactivity. This reagent may be useful for the labeling of hydroxy group in hydroxysteroids. The application for the determination of bio-active substances in biological fluids will be a subject in the future communication.

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