*Journal of Chromatography*, 641 (1993) 176–179
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 172

# Short Communication

# New labelling agent, 2-[2-(isocyanate)ethyl]-3-methyl-1,4-naphthoquinone, for high-performance liquid chromatography of hydroxysteroids with electrochemical detection

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(First received December 1st, 1992; revised manuscript received March 31st, 1993)

ABSTRACT

2-[2-(Isocyanate)ethyl]-3-methyl-1,4-naphthoquinone has been synthesized and developed as a highly sensitive labelling agent for hydroxysteroids in HPLC with electrochemical oxidation after post-column reduction with platinum catalyst. Optimum reaction conditions were examined with cholesterol. The reagent reacted with hydroxysteroids in acetone to give the corresponding carbamic acid esters. The detection limit (signal-to-noise ratio = 3) of cholesterol was 17 fmol as the injected amount. The reagent is suitable for labelling of a relatively unhindered hydroxy group in a hydroxysteroid.

## INTRODUCTION

Various UV or fluorescence derivatization reagents, *e.g.* 3,5-dinitrobenzoyl chloride [1], 1-anthroyl nitrile [2], 7-methoxycoumarin-3- and -4-carbonyl azides [3], pyrene-1-carbonyl nitrile [4], 3,4-dihydro-6,7-dimethoxy-4-methyl-3-oxoquinoxaline-2-carbonyl acid [5], 2-(carboxyphenyl)-5,6-dimethylbenzimidazole [6] and 3-(2-phthalimidyl)benzoyl azide [7] have been reported for the determination of aliphatic alcohols and hydroxysteroids by high-performance liquid chromatography (HPLC). In contrast, only a few reagents for electrochemical detection (ED) have been developed for the derivatization of alcohols [8]. In the previous studies, 2-[2-(azidocarbonyl)ethyl]-3-methyl-1, 4-naphthoquinone (AMQ) used as an ED labelling reagent for hydroxy group was reported [9]. However, the procedure for its derivatization requires a long time (30 min) with cholesterol [10]. Therefore we have studied AMQ analogues to improve the reagent reactivity and developed 2-[2-(isocyanate)ethyl]-3-methyl-1,4-naphthoquinone (IMQ) with isocyanate as a reacting group. This paper describes the preparation of IMQ and the optimum conditions for ED pre-column labelling of hydroxysteroids with IMQ in HPLC.

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0021-9673/93/$06.00 

## EXPERIMENTAL

### *Chemicals*

Steroids were purchased from Sigma (St. Louis, MO, USA). Other chemicals were obtained from Tokyo Kasei (Tokyo, Japan). Organic solvents were distilled and dried prior to use in the usual manner. All other chemicals and solvents employed were of analytical-reagent grade.

Stock solutions (2.5 μmol/ml) of hydroxysteroids were prepared by dissolving each in benzene and diluting to appropriate concentrations prior to use.

### *Apparatus and chromatographic conditions*

The HPLC system used in this work was a Hitachi L-6200 pump (Hitachi, Tokyo, Japan) equipped with a TOA ICA 3060 amperometric detector (Toa Electronics, Tokyo, Japan) with glassy carbon working electrode and a Ag/AgCl reference electrode (applied potential + 0.7 V) and with a Shimadzu CR-6A integrator (Shimadzu, Kyoto, Japan). An Inertsil $C_8$ column (15 cm × 4.0 mm I.D., particle size 5 μm; GL Sciences, Tokyo, Japan) was used at ambient temperature. Methanol–water (95:5) containing 0.05 *M* sodium perchlorate was used as the mobile phase at a flow-rate of 1.0 ml/min. The effluent from the column was directly passed through the platinum catalyst column (10 × 4.6 mm I.D. in stainless-steel column) [9,11]. This column was packed with platinum catalyst (5% on alumina, 10 μm, provided by Toa Electronics) by tapping, and purged with water at a flow-rate of 10 ml/min for 5 min.

Mass spectra were measured with a Hitachi M-1000 LC API mass spectrometer. Proton ($^1H$) nuclear magnetic resonance spectra were recorded on a JEOL JNM-PMX60SI NMR spectrometer with tetramethylsilane (TMS) as an internal standard, and for the infrared spectra a Perkin-Elmer FT-IR 1720 was used.

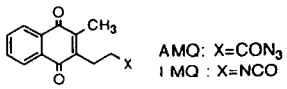


Fig. 1. Structures of labelling reagents.

### *Synthesis of IMQ*

AMQ (500 mg), previously synthesized by the authors [9] (see Fig. 1), was dissolved in 10 ml of benzene. The solution was heated at 100°C for 1 h and then cooled. 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–hexane (1:10). IMQ (400 mg, 89%): m.p. 70–71°C; $^1H$ NMR ($C^2HCl_3$) δ 2.2 (3H, s), 2.9 (2H, t, 7 Hz), 3.5 (2H, t, 7 Hz), 7.5–8.2 (4H, m); IR ($CHCl_3$) $cm^{-1}$ 1598, 1621, 1662, 2276; MS *m/z* 274 ($M^+ + CH_3OH + 1$), 242 ($M^+ + 1$), 199 ($M^+ - NCO$).

### *Chemical analysis of IMQ cholesterol derivative*

IMQ (90 mg, 0.37 mmol) and cholesterol (100 mg, 0.25 mmol) were dissolved in 4 ml of acetone. The solution was placed in a test tube (10 ml), heated at 100°C for 30 min and then cooled to room temperature. The reaction mixture was then evaporated to dryness *in vacuo*, and the residue was dissolved in 2 ml of ethyl acetate and chromatographed on a silica gel C-200 (Wako, Osaka, Japan) with hexane–ethyl acetate (9:1). The main fraction was evaporated to dryness under reduced pressure, and the residue was recrystallized from hexane–ethyl acetate to give IMQ cholesterol derivative as yellow needles (134 mg, 82%): m.p. 167–169°C; $^1H$ NMR ($C^2HCl_3$) δ 0.7 (3H, s), 0.89 (3H, d) 0.95 (6H, d), 1.53 (3H, s), 0.7–2.4 (29H, m), 2.2 (3H, s), 2.8–3.1 (2H, m), 3.2–3.6 (2H, m), 4.6 (1H, m), 5.3 (1H, m), 7.5–8.2 (4H, m); IR ($CHCl_3$) $cm^{-1}$ 1509, 1598, 1660, 1712, 2950, 3019, 3457. MS *m/z* 628 ($M^+ + 1$), 369.

### *Derivatization procedure for HPLC analysis*

A 5-μl aliquot of a test solution of hydroxysteroids in benzene (2.5 μmol/ml each) was placed in a test tube (10 ml), and then 200 μl of 0.1% IMQ in acetone were added. The mixture was heated at 100°C for 15 min. (The solvents were almost distilled off.) After cooling, 1 ml of methanol was added to the mixture, and 10 μl of the reaction mixture were injected into the chromatograph.

## RESULTS AND DISCUSSION

IMQ was readily prepared from AMQ. This chemical structure was confirmed by the mass, $^1$H NMR and IR spectral data. IMQ was stable for more than a year below 0°C. The acetone solution of the reagent was stable for at least a week in a refrigerator.

To investigate the reactivity of IMQ with hydroxysteroids, we have used cholesterol (CE) as a model compound. The derivatization yield was estimated by comparing the peak height with that of the authentic sample. In this study, acetone was found to be suitable for labelling with respect to reactivity in contrast to other solvents, ethyl acetate, dichloromethane, acetonitrile and benzene. The derivatization reaction with CE proceeded at increasing reaction temperature. Constant peak height was attained at a concentration of 0.1% IMQ in the reaction solution at 100°C for 15 min; the reaction yield was 97%. However, at 120°C, the peak height decreased with heating time (Fig. 2). Fig. 3 shows a typical chromatogram of cholesterol and cholestanol derivatized with IMQ. The detection limit ($S/N = 3$) of CE was 17 fmol as the injected amount, with dilution to appropriate concentration of stock solution prior to the derivatization procedure.

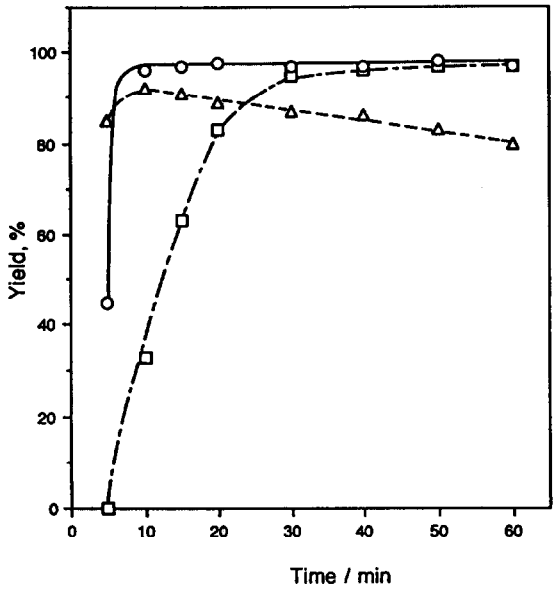


Fig. 2. Effects of reaction time and temperature on IMQ derivatization of cholesterol. □ = 80°C; ○ = 100°C; △ = 120°C.

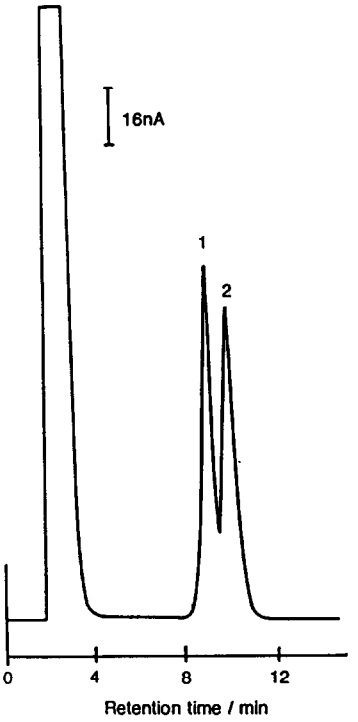


Fig. 3. Chromatogram of IMQ derivatives of cholesterol and cholestanol. A 5-$\mu$l portion of benzene solution (2.5 $\mu$mol/ml each) was treated with IMQ, and 10 $\mu$l (125 pmol each) of the reaction mixture were injected into HPLC as described in the Experimental section. Peaks: 1 = cholesterol; 2 = cholestanol. Mobile phase: 95% methanol (containing 0.05 *M* sodium perchlorate).

The reactivity of IMQ with various hydroxysteroids was investigated under the same conditions. The reaction yields and retention times of the compounds examined under these conditions are shown in Table I. Derivatization of the hydroxy group of C-3 and C-17 proceeded with ease, providing the carbamic esters in 70–97% yield. On the other hand, less reactivity was observed for the 11- and phenolic hydroxy groups. This difference in the reactivity of hydroxysteroids can probably be ascribed to steric hindrance due to the steroid moiety and acidity.

The new reagent has excellent characteristics concerning reactivity and sensitivity for the derivatization of hydroxysteroids, and is more stable than AMQ. Therefore, IMQ should be

TABLE I

RETENTION TIMES AND REACTION YIELDS FOR THE IMQ DERIVATIVES OF HYDROXYSTEROIDS

| Compound | Position of hydroxyl group[a] | Reaction yield (%)[b] | Retention time (min)[c] |
|---|---|---|---|
| 5-Cholesten-3β-ol (cholesterol) | 3β (e) | 97 ± 2 | 8.8 (95) |
| 5α-Cholestan-3β-ol (cholestanol) | 3β (e) | 90 ± 3 | 9.6 (95) |
| 5α-Androstan-3α-ol-17-one (androsterone) | 3α (a) | 78 ± 2 | 25.2 (70) |
| 4-Androsten-17β-ol-3-one (testosterone) | 17β (qe) | 81 ± 3 | 17.0 (70) |
| 4-Androsten-17α-ol-3-one (17α-epitestosterone) | 17α (qa) | 70 ± 3 | 15.2 (70) |
| 4-Pregnene-11α-ol-3,20-dione (11α-hydroxyprogesterone) | 11α (e) | 42 ± 3 | 25.7 (60) |
| 4-Androstene-11β-ol-3,17-dione | 11β (a) | 0 | – |
| 3-Hydroxy-1,3,5 (10)-estratrien-17-one (estrone) | 3 (phen) | 10 ± 2 | 12.2 (70) |

[a] a = axial, e = equatorial, qe = quasiequatorial, qa = quasiaxial, phen = phenolic.
[b] Mean ± S.D. ($n = 4$).
[c] Mobile phase, aqueous methanol (%, v/v).

useful as an ED derivatization reagent in HPLC of a micro amount of hydroxysteroids. We have observed a linear relationship between the peak heights of IMQ-cholesterol and IMQ-cholestanol and reaction amounts up to 10 ng, and a coefficient of correlation ($r$) of 0.998 and 0.981, respectively [10]. These results suggest that the use of this reagent is enough to determine cholesterol and cholestanol in serum. Application to the determination of cholesterol and cholestanol in biological fluids is the subject of a future communication.

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