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NEW FERROCENE REAGENTS FOR DERIVATIZATION OF ALCOHOLS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

New derivatization reagents possessing ferrocene as an electrophore have been developed for high-performance liquid chromatography with electrochemical detection. Among several reagents examined, ferrocenoyl azide and 3-ferrocenylpropionyl azide proved to be satisfactory for use in derivatization of alcoholic hydroxyl compounds with respect to reactivity and sensitivity. Hydroxysteroids were readily condensed with these carboxylic acid azides under mild conditions to provide urethanes, exhibiting maximum sensitivity at +0.4 V *vs.* a silver–silver chloride reference electrode with a detection limit of 0.5 pmol. This derivatization method was found to be applicable to the characterization of the products formed from digoxigenin by *in vitro* bioconversion.

INTRODUCTION

High-performance liquid chromatography (HPLC) is a useful tool for the separation and determination of trace amounts of various compounds in biological fluids. In order to extend the applicability of HPLC, numerous pre- and post-column labelling reagents for UV, fluorescence[1,2] and electrochemical[3–7] detectors have been developed. In a previous paper, we proposed a novel ferrocene reagent for pre-column labelling of amines in HPLC with electrochemical detection (ED)[7]. The ferrocene derivative is readily oxidizable and selectively detected in the presence of other electroactive compounds, such as phenols and aromatic amines.

The present paper deals with the preparation and properties of the derivatization reagents having ferrocene as an electrophore for alcoholic hydroxyl compounds in HPLC–ED. In addition, the application of one of these derivatization reagents to the characterization of the metabolites derived from digoxigenin is also described.

EXPERIMENTAL

Materials

Ferrocene carboxaldehyde was obtained from Aldrich (Milwaukee, WI,

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U.S.A.). Steroids were kindly donated by Teikoku Hormone (Tokyo, Japan). Digitoxigenin monodigitoxoside was prepared from digitoxin by a known method[8]. All other reagents and chemicals were purified by recrystallization or distillation prior to use. Silica gel 60 (Merck, Darmstadt, F.R.G.) was used for column chromatography.

Preparation of derivatization reagents

Melting points were taken on a micro hot-stage apparatus and are uncorrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a JEOL FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviations used are s = singlet and m = multiplet. Low- and high-resolution mass spectra (MS) were taken on Hitachi M-52G and JEOL JMS-01SG-2 spectrometers, respectively. Infra-red (IR) spectra were recorded on a JASCO IRA-1 spectrometer.

Ferrocenoyl cyanide (II)

Trimethylsilyl cyanide (3 ml) was added to a solution of ferrocenoyl chloride (I) (500 mg; obtainable from ferrocene carboxylic acid[9]) and zinc iodide (1 mg) in dichloromethane (10 ml), and the resulting solution was stirred for 3 h[10]. After removal of the precipitate by filtration, the filtrate was evaporated *in vacuo*. The residue was subjected to column chromatography on silica gel (10 cm × 1 cm I.D.). Elution with benzene and recrystallization of the dried eluate from hexane gave compound II (100 mg) as dark red prisms (m.p. 95–97°C). Calculated for $C_{12}H_9FeNO$: C, 60.29; H, 3.80; N, 5.86. Found: C, 60.34; H, 3.58; N, 6.11. High-resolution MS *m/z*: 239.0029 (M^+) (calculated for $C_{12}H_9FeNO$, 239.0032). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2240 (C≡N).

Ferrocenoyl azide (III)

Compound I (600 mg) was treated with sodium azide (160 mg) in the manner described by Arimoto and Haven[9] to give compound III (462 mg) as yellow prisms [m.p. 84–85°C (from diethyl ether); literature m.p. 74–75°C[9]]. High-resolution MS *m/z*: 255.0194 (M^+) (calculated for $C_{11}H_9FeN_3O$, 255.0096). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 2130 (N_3), 1680 (C=O).

3-Ferrocenylpropionyl chloride (IV), 3-ferrocenylpropionyl azide (V)

Phosphorous trichloride (2 ml) was added to a solution of 3-ferrocenylpropionic acid (200 mg) (obtainable from ferrocene carboxaldehyde[7]) in benzene (40 ml), and the mixture was kept at 60°C for 3 h. After removal of the precipitate by filtration, the filtrate was evaporated *in vacuo* to give compound IV as a red oily substance (m.p. < 30°C). Low-resolution MS *m/z*: 276, 278 (M^+). The crude product (200 mg) in acetone (1 ml) was stirred with sodium azide (50 mg) in water (0.1 ml) at 4°C for 45 min. The reaction mixture was poured into cold water, and the precipitate was collected by filtration, washed with water, and dried. The crude product was recrystallized from ether to give compound V (90 mg) as yellow prisms (m.p. 55–56°C). High-resolution MS *m/z*: 283.0396 (M^+) (calculated for $C_{13}H_{13}FeN_3O$, 283.0407). ^{1}H NMR (C^2HCl_3) δ: 2.64 (4H, s, ferrocenyl–CH_2CH_2–), 4.09–4.13 (9H, m, ferrocenyl H). IR ν_{max}^{KBr} cm^{-1}: 2150 (N_3), 1710 (C=O).

High-performance liquid chromatography

The apparatus used for HPLC was a Toyo Soda 803A high-performance liquid chromatograph (Toyo Soda, Tokyo, Japan), equipped with a Yanagimoto VMD-501 electrochemical detector (Yanagimoto, Kyoto, Japan). A TSK gel ODS-120T column (5 μm; 15 cm × 0.4 cm I.D.) (Toyo Soda) was used at a flow-rate of 1 ml/min under ambient conditions.

Derivatization procedures

(i) With acyl chloride. Steroid (1 μg) was added to a solution of compound I or IV (50 μg) in pyridine (1 ml), and the mixture was allowed to stand at 60°C.

(ii) With acyl cyanide. Steroid (1 μg) was added to a solution of compound II (50 μg) in acetonitrile–triethylamine (1:1) (1 ml), and the mixture was allowed to stand at 60°C.

(iii) With acyl azide. Steroid (1 μg) was added to a solution of compound III or V (30 μg) in benzene (1 ml), and the mixture was allowed to stand at 80°C.

An aliquot of the resulting solution was injected into the chromatograph. The derivatization rate was estimated by comparison of the peak height with the authentic sample.

Characterization of the metabolites formed from digoxigenin by HPLC–ED

Male Wistar rats, weighing 150–200 g, were starved overnight before being sacrificed, and 10% liver homogenate was prepared in 0.25 *M* sucrose. Protein was determined by the method of Lowry *et al.*[11] using bovine serum albumin as a reference.

The homogenate (10 μg of protein), nicotinamide-adenine dinucleotide phosphate, reduced (NADPH; 20 μg) in water (0.1 ml), nicotinamide-adenine dinucleotide, oxidized (NAD; 20 μg) in water (0.1 ml), digoxigenin (1 μg) in methanol (0.1 ml) and sufficient 0.05 *M* Tris–HCl buffer (pH 7.4) were used to make a total volume of 2 ml. Incubation was carried out at 37°C for 60 min. After denaturation with heat, digitoxigenin (500 ng), *i.e.* the internal standard (I.S.), was added to the incubation mixture. The mixture was extracted with dichloromethane–ethyl acetate (5:1) (3 × 2 ml), and the organic layer was washed with water (2 × 2 ml) and evaporated under a stream of nitrogen. The residue was treated with compound III (80 μg) in benzene (0.2 ml) at 80°C for 15 min. The product was applied to a silica gel column (3 cm × 0.6 cm I.D.) and eluted with benzene–ethyl acetate (10:1) (5 ml) and then with ethyl acetate (5 ml). The latter eluate was collected and evaporated *in vacuo*. The residue was dissolved in methanol and an aliquot of the solution was subjected to HPLC–ED.

RESULTS AND DISCUSSION

The design of a promising derivatization reagent for alcohols in HPLC–ED requires two structural features: a functional group at least moderately reactive toward the hydroxyl group and an electrophore that is highly responsive to a detector. In the present study, development of the derivatization reagents possessing ferrocene as an electrophore has been undertaken. The structures of the new ferrocene derivatization reagents prepared are shown in Fig. 1.

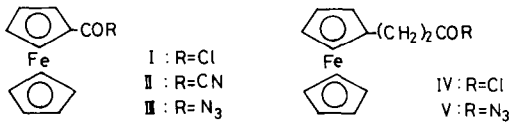

Fig. 1. Structures of ferrocene reagents for use in derivatization of alcohols.

First, we examined the reactions of ferrocenoyl chloride (I) and ferrocenoyl cyanide (II) as the labelling reagents. The reactivities with dehydroepiandrosterone, which was chosen as a model compound, were examined in pyridine at 60°C. The comparative data on the reactivities of ferrocene derivatization reagents are shown in Table I. Unfortunately, these two compounds were not as reactive as expected. The hydrodynamic voltammograms of the resulting ferrocene derivatives indicated that the maximum sensitivity could be obtained at +0.8 V *vs.* a silver–silver chloride reference electrode (Fig. 2). At this applied potential, other electroactive compounds, such as phenols, guaiacols and aromatic amines, are also responsive to the detector[12]. It seems likely that direct linking of the electron-withdrawing carbonyl group to the electrophore would depress their reactivities and exert the anodic shifts of the resulting derivatives. This assumption was supported by inspection of the properties

TABLE I

REACTIVITIES OF FERROCENE DERIVATIZATION REAGENTS WITH DEHYDROEPIANDROSTERONE

Figures express the yield (%) of corresponding derivatives.

Reaction time (min)	*Reagent*				
	I	*II*	*III*	*IV*	*V*
10	10	5	100	50	100
60	30	15	100	100	100

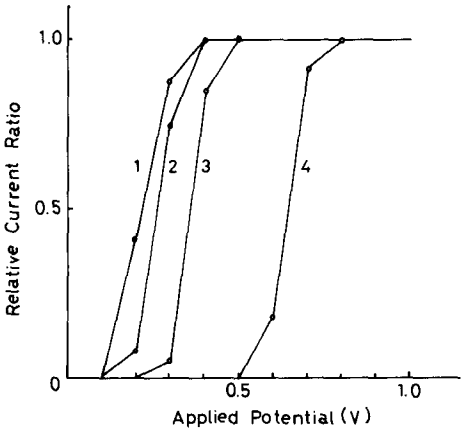

Fig. 2. Hydrodynamic voltammograms of dehydroepiandrosterone derivatives, produced with ferrocene reagents (I–V). (1) III, (2) V, (3) IV, (4) I or II.

of 3-ferrocenylpropionyl chloride (IV). Indeed, derivatization was completed in a short period and the half-wave potential of the product was +0.36 V (Fig. 2). However, compound IV is not fully suitable as a derivatization reagent, because it melts at low temperature (< 30°C) and decomposes gradually with moisture.

These results prompted us to prepare carboxylic acid azides (III, V), which are capable of reacting with alcohols to give urethanes[13] (Fig. 3). The reagents were easily obtained as yellow crystals from the corresponding carboxylic acid chloride. They are stable for at least six months, if kept at 4°C. Their reactivities and the electrochemical properties of the resulting derivatives were then investigated. Both reagents gave stable derivatives when treated in benzene at 80°C for 10 min.

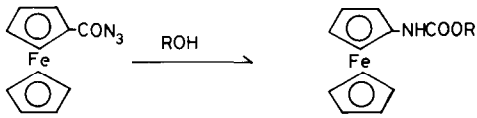

Fig. 3. Reaction of alcohols with ferrocenoyl azide.

The hydrodynamic voltammograms of the resulting urethanes indicated that the maximum sensitivity could be obtained at +0.4 V *vs.* a silver–silver chloride reference electrode, where common electroactive compounds would provide no response. The collection efficiency of these derivatives was estimated to be 0.28 (upstream +0.5 V, downstream 0.0 V), which is higher than that of catechols (0.26). No significant difference in reactivity and electrochemical properties was observed between the two compounds. The reactivity of compound III with various steroidal alcohols was investigated under the standard conditions. The results obtained are listed in Table II. No distinct difference was seen in the reactivity among primary, phenolic, axial and equatorial hydroxyl groups. The 12β-hydroxyl group of digoxigenin, which is sterically more hindered than the 3β-hydroxyl group, was also derivatized with ease. The reaction with the sugar alcohols in digitoxigenin monodigitoxoside was complete in 30 min. The detection limit of the dehydroepiandrosterone derivative was 0.5 pmol (signal-to-noise ratio = 5 at 4 nA full scale) and multiple ferrocene substituents proportionally increased the response to the detector.

TABLE II

REACTIVITIES OF FERROCENOYL AZIDE WITH VARIOUS HYDROXYL GROUPS

Compound	*Position of hydroxyl group**		*Reaction time (min)***
Dehydroepiandrosterone	3β	(eq)	10
Methyl lithocholate	3α	(eq)	10
5β-Cholane-3α,24-diol	3α, 24	(eq, prim)	10
Estrone	3	(phen)	10
Digitoxigenin	3β	(ax)	10
3-Epidigitoxigenin	3α	(eq)	10
Digoxigenin	3β, 12β	(ax, eq)	10
Digitoxigenin monodigitoxoside	3′, 4′	(ax, eq)	30

* Prim = primary, phen = phenolic, ax = axial, eq = equatorial.

** Reaction time required for 100% derivatization.

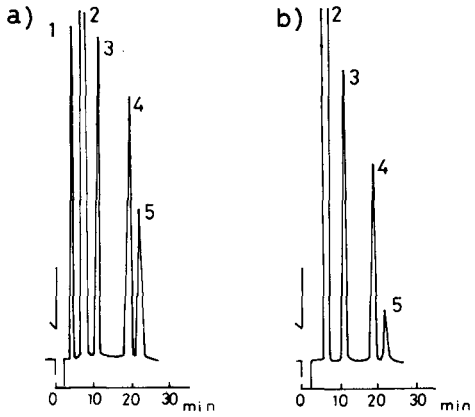

Fig. 4. Chromatograms of cardiac steroids. (a) A synthetic mixture of authentic samples; (b) bioconversion products formed from digoxigenin by incubation with the rat liver homogenate. Peaks: 1 = 3-dehydrodigoxigenin, 2 = ferrocene reagent, 3 = digitoxigenin (I.S.), 4 = digoxigenin, 5 = 3-epidigoxigenin. Conditions: TSK gel ODS-120T column (5 μm; 15 cm × 0.4 cm I.D.); mobile phase, acetonitrile–water (3:2) containing 0.1 *M* $NaClO_4$, flow-rate, 1 ml/min.

The utility of compound III was tested for the characterization of metabolites from digoxigenin formed by the rat liver homogenate. It has been established that digoxigenin, one of the major metabolites of digoxin, undergoes further biotransformation[14], but the details still remain unclear in the absence of a reliable analytical method. On treatment with compound III, the authentic 3-dehydrodigoxigenin, digitoxigenin (I.S.), digoxigenin and 3-epidigoxigenin were quantitatively derivatized in 15 min. The resulting products were distinctly separated by HPLC on TSK gel ODS-120T, as illustrated in Fig. 4a. Digoxigenin was incubated with the enzyme preparation and the incubation mixture was processed by the standard procedure. A typical chromatogram exhibited two peaks, assignable to the 3-epidigoxigenin formed and to unchanged digoxigenin, without any interfering peaks (Fig. 4b). When the ratio of the amount of each cardiac steroid to the I.S. (250 ng) was plotted against peak-height ratio, a linear relationship was observed in the range 25–1000 ng/ml. In addition, cardiac steroids, added to the denatured standard incubation medium at two levels (250, 500 ng/ml), were recovered at the rate of > 76.4% (coefficient of variation < 5%; $n = 7$). It is evident from these data that the proposed analytical procedure is satisfactory, both in accuracy and precision.

In conclusion, the newly developed derivatization method using compound III or V has proved to be satisfactory for HPLC–ED of alcohols with respect to selectivity and sensitivity. The derivatization reagents are stable and moderately reactive and, hence, suitable for the determination of various bioactive substances (such as hydroxysteroids) in biological fluids. The biomedical applications of the present method are being conducted in these laboratories, and details will be reported elsewhere.

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