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New derivatization method using ferrocene reagents for the determination of steroid glucuronides by high-performance liquid chromatography with electrochemical detection*

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Abstract: New derivatization methods using ferrocene reagents have been developed for the determination of steroid glucuronides by high-performance liquid chromatography with electrochemical detection. Condensation of glucuronides with 2-ferrocenylethylamine is effected in the presence of water-soluble carbodiimide and 1-hydroxybenzotriazole. The resulting amides show the satisfactory sensitivity at +0.45 V versus Ag/AgCl reference electrode, with a detection limit of 0.5 pmole. The present method is found to be applicable to the estimation of estrogen glucuronyltransferase activities and estrogen glucuronides in pregnancy urine.

Keywords: *2-Ferrocenylethylamine; estrogen glucuronide; pre-column derivatization; electrochemical detection; high-performance liquid chromatography (HPLC).*

Introduction

High-performance liquid chromatography (HPLC) is a useful tool for the trace analysis of various compounds in biological fluids. In order to extend its applicability, numerous pre- and post-column labeling reagents have been developed for use with ultraviolet, fluorescence [1, 2] and electrochemical detectors [3–8]. In the previous papers of this series novel ferrocene reagents have been introduced for pre-column labeling of amino [7] and hydroxyl compounds [8] in HPLC with electrochemical detection (ECD). The ferrocene derivatives are readily oxidized and hence, can be detected selectively in the presence of other electroactive compounds, such as catechols, phenols and aromatic amines.

The present paper deals with the preparation and properties of derivatization reagents having ferrocene as an electrophore for the determination of estrogen glucuronides by HPLC–ECD. In addition, the application of this method to the estimation of estrogen glucuronyltransferase activities and estrogen glucuronides in pregnancy urine also is described.

*Part CCXXIV of *Studies on Steroids* by T. Nambara. Following abbreviations were used in this paper: estrone (E_1), estradiol (E_2), estriol (E_3) and glucuronide (G).

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Experimental

Materials

Ferrocene and *N,N*-dimethylaminomethylferrocene were obtained from Aldrich Chemical Co. (Milwaukee, WIS, USA). 1,1′-Dimethylferrocene was purchased from Tokyo Kasei Co. (Tokyo, Japan) and purified by chromatography on TOYO PAK ODS column (Toyo Soda Co., Tokyo, Japan) to remove the contaminant monomethylferrocene. Steroids were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo, Japan). Estrogen glucuronides were synthesized in these laboratories as described in the previous paper [9]. All other reagents and chemicals were purchased from Nakarai Chem. Co. (Kyoto, Japan) and purified by recrystallization or distillation prior to use. Sep-Pak C_{18} cartridge was supplied by Waters Assoc. (Milford, MA, USA). Silica gel 60 and HF_{254} (E. Merck AG, Darmstadt, FRG) were used for column chromatography and thin layer chromatography (TLC), respectively.

Instruments

Melting points were taken on a micro hot-stage apparatus (Yanagimoto Co., Kyoto, Japan) 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 (IS). Abbreviations used are s = singlet and m = multiplet. Low- and high-resolution mass (MS) spectral measurements were performed using Hitachi M-52G and JEOL JMS-01SG-2 spectrometers, respectively.

HPLC was carried out on a Toyo Soda 803A chromatograph (Toyo Soda Co.) equipped with a Yanagimoto VMD-501 electrochemical detector (Yanagimoto Co.). The applied potential was set versus an Ag/AgCl reference electrode. A TSKgel ODS-80TM (5 μm) column (15 cm × 0.4 cm i.d.) (Toyo Soda Co.) was used under ambient conditions.

Preparation of bromoacetylferrocene (I)

Monobromoacetyl chloride (0.97 g) in anhydrous methylene chloride (2 ml) was added dropwise to a solution of ferrocene (1 g) in anhydrous methylene chloride (13 ml) under N_2 gas stream and ice-cooling. Anhydrous $AlCl_3$ (0.86 g) was added portion wise to the solution, which was then stirred for 1 h under the same conditions. The reaction mixture was poured into ice-water and extracted with methylene chloride. The organic layer was washed with 5% aqueous $NaHCO_3$ and dried over anhydrous Na_2SO_4. After evaporation of the organic solvent, the dark red oily residue was subjected to column chromatography (30 cm × 1.5 cm i.d.) and eluted with benzene. The dried eluate was recrystallized from ether to give I (100 mg) as red prisms, m.p. 65–70°C. High resolution MS *m/z*: 305.9320, 307.9300 $(M)^+$ (calculated for $C_{12}H_{11}BrFeO$: 305.9342, 307.9322).

Preparation of 3-bromoacetyl-1,1′-dimethylferrocene (II) *and 2-bromoacetyl-1,1′-dimethylferrocene* (III)

1,1′-Dimethylferrocene was treated with monobromoacetyl chloride (450 mg) and $AlCl_3$ (400 mg) in the manner described above. The residue obtained was subjected to column chromatography (30 cm × 1.5 cm i.d.) using benzene as an eluant. The first red eluate was recrystallized from methanol to give III (5 mg) as red prisms, m.p. <30°C. High resolution MS *m/z*: 333.9658, 335.9654 $(M)^+$ (calculated for $C_{14}H_{15}BrFeO$: 333.9656, 335.9636). ^{1}H-NMR ($CDCl_3$)δ: 1.89 (3H, s, 1′-CH_3), 2.29 (3H, s, 1-CH_3),

3.95–4.10 (4H, m, 2′~5′-H), 4.22 (2H, ABq, CH_2Br), 4.38–4.58 (3H, m, 3~5-H). The subsequent red eluate was recrystallized from methanol to give II (30 mg) as red prisms, m.p. 37–40°C. High resolution MS *m/z*: 333.9632, 335.9623 $(M)^+$ (calculated for $C_{14}H_{15}BrFeO$: 333.9656, 335.9636). ^{1}H-NMR ($CDCl_3$)δ: 1.87 (3H, s, 1′-CH_3), 2.05 (3H, s, 1-CH_3), 4.02 (4H, br s, 2′~5′-H), 4.18 (2H, s, CH_2Br), 4.42 (1H, br s, 5-H), 4.65 (2H, m, 2-, 4-H).

Preparation of 2-ferrocenylethylamine (IV)

The compound IV was obtained from *N,N*-dimethylaminomethylferrocene as a red oily substance by the method of Lednicer *et al.* [10]. The product was stable for at least 3 months when dissolved in chloroform and kept at 4°C. Hydrogen chloride gas was passed into a solution of IV in ether to give a crystalline product which in turn was recrystallized from methanol–acetone to give the hydrochloride of IV as red prisms, m.p. 200–205°C (dec.). Elemental analysis is as follows. Calculated for $C_{12}H_{15}FeN{\cdot}HCl$: C, 54.19; H, 6.06; N, 5.27; Cl, 13.48; Found: C, 53.84; H, 5.99; N, 4.96; Cl, 13.41.

Preparation of authentic estrogen glucuronide derivatives with IV

Estrogen glucuronide (5 mg) dissolved in pyridine (0.5 ml) and chloroform (2 ml) was treated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (water-soluble carbodiimide, WSC) (10 mg), IV (10 mg) and 1-hydroxybenzotriazole (HOBT) (1 mg) at 37°C for 12 h [11]. The whole was extracted with ethyl acetate, which was washed successively with 5% HCl, 5% aqueous $NaHCO_3$ and dried over anhydrous Na_2SO_4. The solvent was evaporated off and the residue was purified by preparative TLC using chloroform–methanol–H_2O (80:20:2.5, v/v/v) to give the desired compound as a yellow oily substance (*ca* 2 mg). The *Rf* values in TLC [solvent: chloroform–methanol–H_2O (80:20:2.5, v/v/v)] and MS spectral data were as follows: From E_1 3-G, *Rf* 0.77, MS *m/z*: 658 $(M + H)^+$; from E_2 3-G, *Rf* 0.70, *m/z*: 659 $(M)^+$; from E_2 17-G, *Rf* 0.70, *m/z*: 659 $(M)^+$; from E_3 16-G, *Rf* 0.60, *m/z*: 676 $(M + H)^+$; from 16-epiE_3 17-G, *Rf* 0.60, *m/z*: 676 $(M + H)^+$.

Derivatization of estrogen glucuronides with IV

To a solution of estrogen glucuronide (1 μg) in pyridine (40 μl) were added IV (50 μg) in chloroform (50 μl), WSC (400 μg) in chloroform (0.4 ml) and HOBT (10 μg) in pyridine–chloroform (2:3, v/v) (25 μl), and the whole was kept at 37°C for 2 h. The reaction mixture was extracted with ethyl acetate, which was washed successively with 5% HCl, 5% aqueous $NaHCO_3$ and H_2O. After evaporation of the solvent, the residue dissolved in benzene–ethyl acetate–methanol (2:1:0.1, v/v/v) (2 ml) was subjected to chromatography on a silica gel column (3 cm × 0.5 cm i.d.). After elution with benzene (3 ml) and ethyl acetate (3 ml), the desired compound was eluted with ethyl acetate–methanol (4:1, v/v) (3 ml). An aliquot of the solution was used for HPLC. The derivatization rate was estimated by comparison of the peak area with the authentic sample.

Estimation of estrogen glucuronyltransferase activities by HPLC–ECD

Male Wistar rats (250–280 g), Hartley guinea pigs (805–950 g) and an albino rabbit (6 kg) were starved overnight before use. Animals were sacrificed and 10% liver homogenate was prepared in 0.25 M sucrose. Protein was determined by the method of Lowry *et al.* [12] using bovine serum albumin as a reference. The homogenate (3 mg protein), uridine diphosphate glucuronic acid (0.8 mg) in H_2O (0.8 ml), substrate (E_2: 50

μg) in methanol (0.15 ml) and sufficient 0.05 M Tris·HCl buffer (pH 7.45) were used to make the total volume of 4.5 ml, which was then incubated at 37°C. Portions (0.5 ml) of the incubation mixture were taken at 15, 30 and 60 min intervals. After addition of E_3 16-G (IS) (1 μg), the whole was denatured by heating and then centrifuged at 2800 rpm for 10 min. The supernatant was injected onto a Sep–Pak C_{18} cartridge, washed with H_2O (5 ml) and eluted with 50% methanol (5 ml). The eluate was evaporated off under reduced pressure, the residue was derivatized with IV and then subjected to HPLC–ECD in the manner described above.

Determination of estrogen glucuronides in pregnancy urine by HPLC–ECD

16-EpiE_3 17-G (IS) (1 μg) was added to pregnancy urine (0.1 ml) (32–40 weeks of gestation), which was then diluted with H_2O (5 ml) and injected onto a Sep–Pak C_{18} cartridge. The eluate with 50% methanol (5 ml) was evaporated down and the residue was derivatized with IV and then subjected to HPLC–ECD.

Recovery test for estrogen glucuronides added to the incubation medium or control urine

Spiked samples were prepared by adding 1 μg and 250 ng each of the estrogen glucuronides to the incubation medium (0.5 ml) or charcoal-treated control female urine (0.1 ml). A sample was diluted with H_2O (5 ml) and subjected to clean-up with a Sep–Pak C_{18} cartridge, derivatization with IV and HPLC.

Results and Discussion

The determination of steroid glucuronides in biological fluids is one of the more important subjects in clinical chemistry. Several methods involving radioimmunoassay (RIA) [13, 14] or HPLC [15] have been developed. The former are not suitable for profile analysis while the latter is only applicable to the compounds possessing either a chromophore, fluorophore, or electrophore. To the best of the authors' knowledge only one attempt has been made so far to transform these glucuronides into fluorescent derivatives [16]. The design of a promising derivatization reagent for the analysis of glucuronides by HPLC–ECD requires certain structural features namely, a functional group reactive toward the glucuronyl moiety and a highly responsive electrophore. In the present study, the development of the derivatization reagents possessing ferrocene as an electrophore has been undertaken. The structures of newly developed ferrocene derivatization reagents prepared are shown in Fig. 1.

First, the electrochemical properties of bromoacetylferrocene (I), obtained from ferrocene by Friedel–Crafts reaction, have been examined. The hydrodynamic voltammogram of I indicated that the maximum sensitivity could be obtained at +0.70 V. At this applied potential, other electroactive compounds, such as catechols, phenols and aromatic amines showed response to the detector. It is likely that an electron-

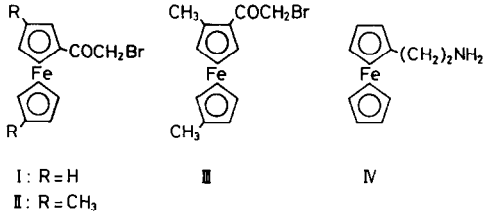

Figure 1
Structures of ferrocene reagents for use in derivatization of glucuronides. I: Bromoacetyl ferrocene, II: 3-bromoacetyl-1,1′-dimethylferrocene, III: 2-bromoacetyl-1,1′-dimethylferrocene, IV: 2-ferrocenylethylamine.

withdrawing carbonyl group directly attached to the electrophore would exert the anodic shift of the resulting derivative [8].

Accordingly it was decided to prepare 2- and 3-bromoacetyl-1,1′-dimethylferrocenes (III, II), which have two electron-donating methyl groups in their sandwich structures, from 1,1′-dimethylferrocene. The structures of these compounds were characterized on the basis of ^{1}H-NMR spectral data. The chemical shift of the 1-methyl group of III was observed at 2.29 ppm, which was *ca* 0.2 ppm downfield from that of 1,1′-dimethylferrocene, as a result of the anisotropy effect of the adjacent bromoacetyl group. Compound II exhibited a half-wave potential ($E_{1/2}$) at +0.48 V, which was lower than that of I (+0.64 V). The reaction of II with E_2 3-G in the presence of the crown ether was attempted, but the desired derivative could not be obtained. Under the same conditions, however, cholic acid was quantitatively transformed into the derivative which showed $E_{1/2}$ at +0.48 V. Accordingly this reagent may be useful for the derivatization of bile acids.

Further work was directed to the use of 2-ferrocenylethylamine (IV) as a derivatization reagent for the steroid glucuronides. The reagent was obtained from *N,N*-dimethylaminomethylferrocene. The reactivity of the reagent and sensitivity of the product were investigated using E_2 3-G as a model compound. Condensation of the glucuronide in pyridine with the reagent was effected in the presence of WSC and HOBT. The yield of the product was estimated by comparison with the peak area of the standard sample. The reaction rate increased with reaction time up to 60 min and reached a plateau. Other estrogen glucuronides (E_1 3-G, E_2 17-G, E_3 16-G, 16-epiE_3 17-G) were also derivatized within 120 min. The detection limit of these glucuronide derivatives at +0.45 V was 0.5 pmole (signal-to-noise ratio = 5 at 4 nA full scale). The hydrodynamic voltammograms of E_2 3-G and E_2 17-G derivatives are shown in Fig. 2. The E_2 17-G derivative having two electrophores, the ferrocenyl and phenolic groups, provided a characteristic voltammogram which would be useful for the identification of peaks in HPLC.

The utility of IV was tested for the estimation of estrogen glucuronyltransferase activities of the rat, guinea pig and rabbit liver homogenates. On being treated with IV, the authentic E_1 3-G, E_2 3-G, E_2 17-G and E_3 16-G (IS) were quantitatively derivatized in 120 min. The resulting products were fully separated by HPLC on TSKgel ODS-80TM as illustrated in Fig. 3a. E_2 was incubated with the enzyme preparation and the incubation mixture was processed by the standard procedure. A typical chromatogram obtained by the use of the guinea pig liver homogenate that exhibits two peaks assignable to the E_2 3-G and E_2 17-G produced and the absence of interfering peaks is shown in Fig. 3b. When the quantitative ratio of each glucuronide to the IS (100 ng) was plotted against the peak height ratio, a linear relationship was observed in the range 12.5–400 ng (injected amount) of the glucuronide, the regression equations being $y = ax + b$ where a = 1.40, 1.38, 1.25 and 0.76 for E_3 16-G, E_2 3-G, E_1 3-G and E_2 17-G, respectively, and $b = 0$. In addition, estrogen glucuronides added to the denatured standard incubation medium at two levels (500 ng, 2 μg/ml) were recovered at the rate of ≥79.6% (RSD <5%, n = 8). It is evident from these data that the proposed analytical procedure is satisfactory with respect to its accuracy and precision.

The formation of E_2 3-G and E_2 17-G was initially examined as a function of incubation time and enzyme amount. The yield of both glucuronides increased linearly with the incubation time up to 30 min. In each run the amounts of glucuronides increased proportionally with the quantity of the enzyme preparation, up to 2.7 mg protein/ml (incubation medium). The specific activities of estrogen glucuronyltransferase assayed

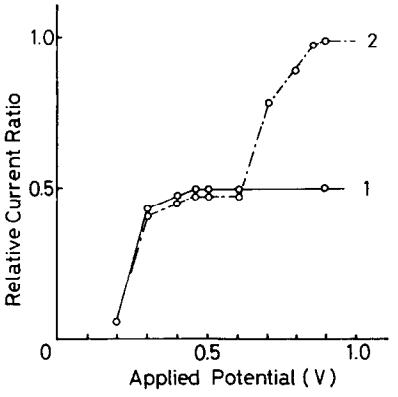

Figure 2
Hydrodynamic voltammograms for E_2 3-G and E_2 17-G derivatives obtainable with 2-ferrocenylethylamine. 1: E_2 3-G, 2: E_2 17-G.

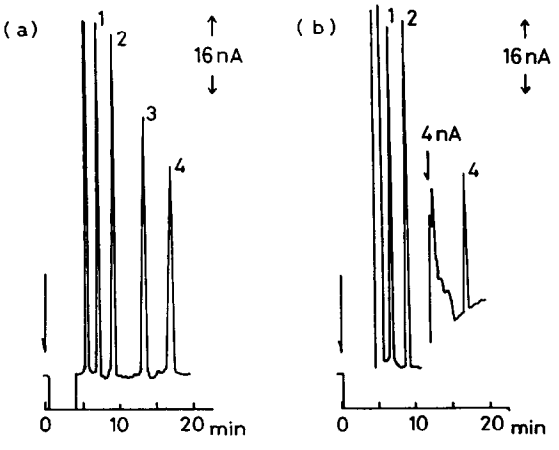

Figure 3
Chromatograms of estrogen glucuronides from incubation medium. (a) A synthetic mixture of authentic samples; (b) bioconversion products from estradiol with the guinea pig liver homogenate. 1: E_3 16-G (IS), 2: E_2 3-G, 3: E_1 3-G, 4: E_2 17-G. Conditions: mobile phase, 0.05 M $NaClO_4$ in acetonitrile–H_2O (9:10, v/v). Flow rate, 1 ml/min. Pre-column derivatization with IV was carried out in the manner described in the text.

for the rat, guinea pig and rabbit liver homogenates under standard conditions are listed in Table 1. The species differences observed have been reported previously [17].

The present method was then applied to the determination of estrogen glucuronides in pregnancy urine. One-tenth milliliter of urine specimen was treated in the manner employed for the incubation study. The satisfactory recovery rates were also obtained as described above. The two peaks corresponding to E_3 16-G and E_1 3-G were observed on a chromatogram (Fig. 4b). It is evident from the data in Table 2 that the results obtained by HPLC–ECD were compatible with those by RIA [13, 14].

The assay of steroid glucuronyltransferase activities is usually performed by the radiometric method [17]. This procedure, however, has inevitable disadvantages with respect to versatility and simplicity owing to the use of a radioactive tracer. The ultraviolet detector is not necessarily suitable for the determination of enzymatically formed estrogen glucuronides [15]. In our previous study, the utility of HPLC–ECD was

Table 1
Specific activities of estradiol glucuronyltransferase

Enzyme source	Product*	
	E_2 3-G	E_2 17-G
Guinea pig†	350	17
Rabbit‡	200	n.d.
Rat†	n.d.	n.d.

*pmole/mg protein·min, †$n = 3$, ‡$n = 1$.
n.d.: not detectable.

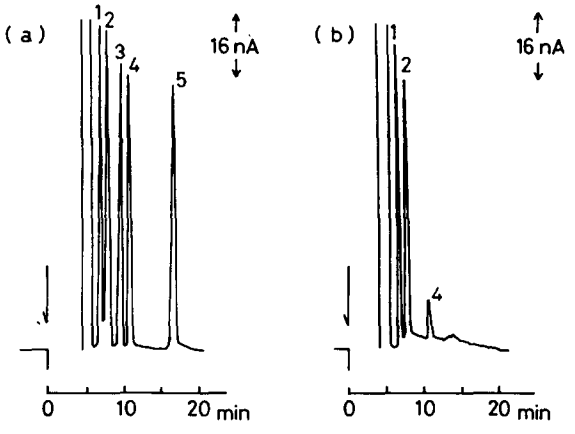

Figure 4
Chromatograms of estrogen glucuronides from pregnancy urine. (a) A synthetic mixture of authentic samples; (b) urine sample. 1: E_3 16-G, 2: 16-epiE_3 17-G (IS), 3: E_2 3-G, 4: E_1 3-G, 5: E_2 17-G. Conditions: mobile phase, 0.05 M $NaClO_4$ in methanol–acetonitrile–H_2O (4:1:20, v/v/v). Flow rate, 1 ml/min. Pre-column derivatization with IV was carried out in the manner described in the text.

Table 2
Levels of E_1 3-G and E_3 16-G in pregnancy urine

No.	Gestation period in weeks	E_1 3-G		E_3 16-G	
		HPLC	RIA	HPLC	RIA
1	32	250*	236	1500*	1700
2	34	250	236	2250	1900
3	35	250	224	2250	1900
4	39	132	112	3850	3600
5	37	333	300	5000	5280
6	40	150	148	2600	2400
7	37	93	88	2330	2360
8	33	149	180	2900	2800
9	40	60	48	2130	2200
10	38	46	64	1620	1480

*ng/0.1 ml.

demonstrated for the determination of estrogen conjugates in pregnancy urine [15]. However, this method is only applicable to the compounds having a free phenolic group at applied potential of above +0.8 V.

The developed derivatization method using 2-ferrocenylethylamine has proved to be satisfactory for the determination of estrogen glucuronides in biological fluids with respect to selectivity and sensitivity. Application of the present method to the estimation of other steroid glucuronides in biological fluids and glucuronyltransferase activities is being conducted in these laboratories and the details will be reported elsewhere.

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