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Short Communication

Determination of estrogens in plasma by high-performance liquid chromatography after pre-column derivatization with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole

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ABSTRACT

A sensitive method for the determination of estrogens by high-performance liquid chromatography with fluorimetric detection was reported. The estrogens were derivatized with 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole to their esters in the presence of 4-piperidinopyridine and 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate. The resulting esters were extracted with a Sep-Pak C_{18} plus cartridge, and then the esters were separated on a Wakosil $5C_{18}$ column with water–methanol (10:90, v/v) as the mobile phase. The esters were detected by fluorimetric detection (excitation wavelength = 336 nm, emission wavelength = 440 nm). The within-day relative standards deviations (n = 6) for each estrogen (1.0 ng per 100 μl of plasma) were 8.7–13.0%, and day-to-day relative standard deviations (n = 6) were 8.3–11.8%. The limits of detection for estrogens (estrone, estradiol, estriol, estetrol, ethynylestradiol, 2-hydroxyestradiol, 4-hydroxyestradiol) were 0.1–0.2 pg per 100 μl of plasma (signal-to-noise ratio 3).

INTRODUCTION

Estrogens are widely determined for monitoring of pregnancy [1], foetal viability [2–4], breast cancer [5–7], uterine corpus cancer [8], prostate cancer [9] and osteoporosis [10–12]. An equine estrogen, ethynylestradiol, has been used as a drug in the treatment of hormone diseases [13,14]. Radioimmunoassay (RIA) [1,10–12], enzyme immunoassay (EIA) [2,7], receptor-binding assay [5,6,8], high-performance liquid chromatography (HPLC) with photometric detection [3,4,9,13,14] or electrochemical detection (ED) [15], gas chromatography (GC) [16] and gas chromatography–mass spectrometry (GC–MS) [17,18] have been used for the determination of estrogens in urine, blood and tissue extracts. HPLC methods are simple, specific, do not require expensive apparatus or radioactive reagents and can determine estrogens simultaneously. HPLC methods after fluorimetric [19] and chemiluminescent [20] pre-column derivatization were developed to improve specificity and sensitivity. These methods can determine picogram amounts of estrogens. We have previously reported a fluorimetric pre-column HPLC method

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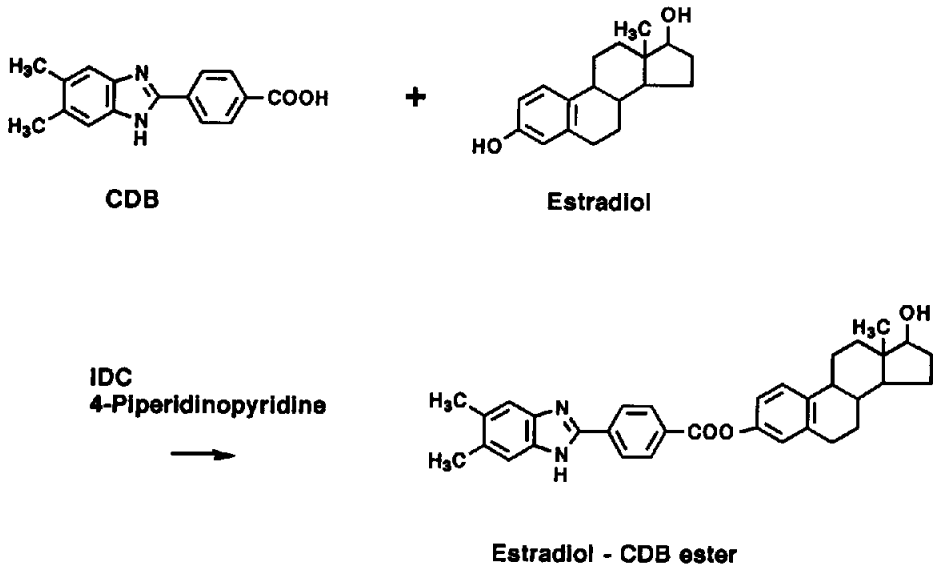

Fig. 1. Derivatization reaction of CDB with estradiol.

for fatty alcohols [21] and corticosteroids [22] using 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB). It can determine fatty alcohols and 21-hydroxycorticosteroid in amounts as low as 0.06–1.0 pg. The CDB reagent is stable and gives fluorescent esters of aliphatic alcohols in the presence of 4-piperidinopyridine and 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate under mild condition. Phenols were also reacted by this method [23–25]. Therefore, we applied the proposed HPLC–CDB method to determine estrogens. In this study, the derivatization reaction proceeded as shown in Fig. 1.

EXPERIMENTAL

Sample and materials

Estrogens (estrone, estradiol, estriol, estetrol, 2-hydroxyestradiol, 4-hydroxyestradiol, ethynylestradiol, equilin and equilenin; Fig. 2) were purchased from Sigma (St. Louis, MO, USA) and Steraloids (Wilson, NH, USA). A stock solution of steroid (1000 μg/ml was prepared by dissolving 10 mg of steroid in 1 ml of N,N-dimethylformamide and diluting to 10 ml with acetonitrile. CDB was synthesized according to a previous report [21]. CDB (0.3%, w/v) solution was prepared by dissolving 30 mg of the reagent in 3 ml of N,N-dimethylformamide and adding 700 mg of 4-piperidinopyridine (Aldrich, Milwaukee, WI, USA) and then diluting to 10 ml with acetonitrile. This solution was stable for four days in daylight at room temperature. The 1-isopropyl-3-(3-dimethylaminopropyl)carbodiimide perchlorate (IDC) solution (7%, w/v) was prepared by dissolving 700 mg of the reagent (Kanto, Tokyo, Japan) in 10 ml of acetonitrile. This solution was stable for eight days at room temperature. Other reagents were of reagent grade.

Apparatus and HPLC conditions

HPLC apparatus and conditions were as follows: pump, Shimadzu LC-6A liquid chromatograph (Shimadzu, Kyoto, Japan); guard column, Wakosil $5C_{18}$ (50 mm × 4.0 mm I.D., 5 μm; Wako, Osaka, Japan); analytical column, Wakosil $5C_{18}$ (300 mm × 4.0 mm I.D.), 5 μm); sample solvent, 20 μl; column temperature, room temperature (about 22°C); detector, Shimadzu RF-535 fluorescence spectromonitor (excitation wavelength = 336 nm, emission wavelength = 440 nm); mobile phase, water–methanol (10:90, v/v); flow-rate, 0.7 ml/min.

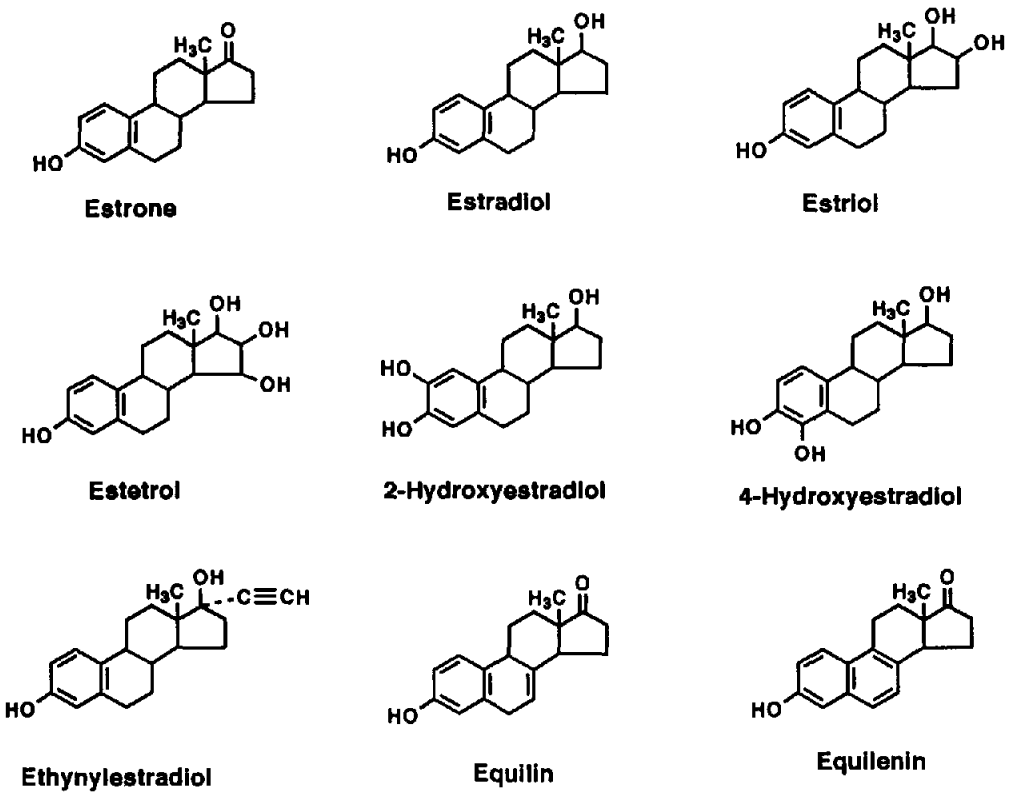

Fig. 2. Structures of estrogens.

Pre-column derivatization of estrogens

The derivatization was performed as described in a previous paper [22]. The only difference was heating at 50°C for 15 min.

Extraction of estrogens from plasma

Plasma (100 μl, containing 1.0 ng of estrone, estradiol, estriol, estetrol, 2-hydroxyestradiol, 4-hydroxyestradiol and ethynylestradiol) was mixed with 10 μl of internal standard (I.S.) (*p*-hydroxybenzoic acid *sec.*-butyl ester in water) and extracted as described previously [22].

RESULTS AND DISCUSSION

Pre-column derivatization

We used estradiol as a model estrogen compound for development of a pre-column HPLC method using CDB. The study of pre-column conditions was carried out similar to a previous report [22]. However, the derivatization process could be carried out at lower temperature.

Extraction of CDB derivatives

The CDB derivatives of estrogens could be extracted by Sep-Pak C_{18} plus cartridges (Waters, Milford, MA, USA) under the same conditions as previously described for 21-hydroxycorticosteroids [22].

Separation of CDB derivatives

The separation of CDB esters of estrogens (estrone, estradiol, estriol, estetrol, 2-hydroxyestradiol, 4-hydroxyestradiol, ethynylestradiol, equilin and equilenin) was studied. Zorbax ODS (7 μm; DuPont, Wilmington, DE, USA), LiChrosorb RP-18-5 (5 μm; Merck, Darmstadt, Germany), Nucleosil 120-5C_{18} (5 μm; Macherey Nagel, Düren, Germany), Wakosil 5C_{18}, 5C_{18}N and 5C_{18}HG (5 μm; Wako, Osaka, Japan) with ace-

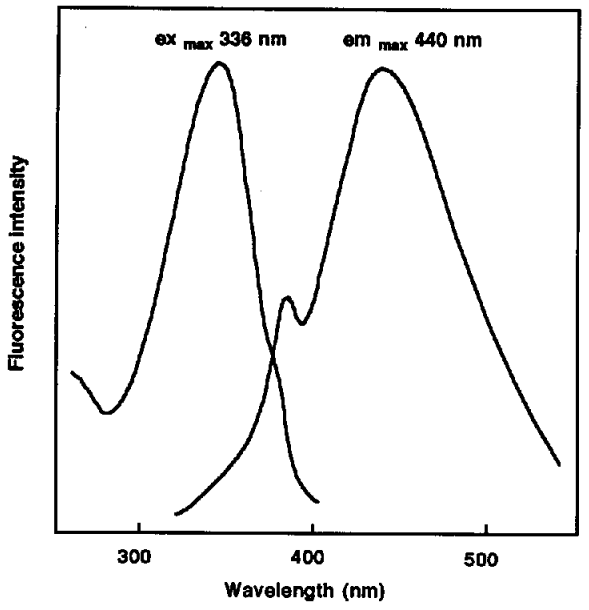

Fig. 3. Excitation and emission spectra of CDB derivative of estradiol in HPLC eluent. Concentration of estradiol used = 100 ng/ml.

tonitrile, tetrahydrofuran, methanol and propan-2-ol as mobile phases were tested. It was found that Wakosil $5C_{18}$ (300 mm × 4.0 mm I.D., 5 μm) with water–methanol (10:90, v/v) as mobile phase was the most suitable for the separation of each estrogen. However, equilin and equilenin were not separated under these conditions. The 21-hydroxycorticosteroids, *e.g.* cortisone, prednisolone and triamcinolone, were eluted within 10 min, so these corticosteroids did not interfere the determination of estrogens.

Excitation and emission spectra of 100 ng/ml estradiol are shown in Fig. 3. The excitation maximum was at around 336 nm, and the emission maximum was at around 440 nm for each estrogen. Therefore, each estrogen was detected at 336 nm for excitation and 440 nm for emission.

A chromatogram of a 100 ng/ml solution of nine estrogens is shown in Fig. 4. The efficiency of the derivatization was studied with estradiol by comparing the detector response given by the

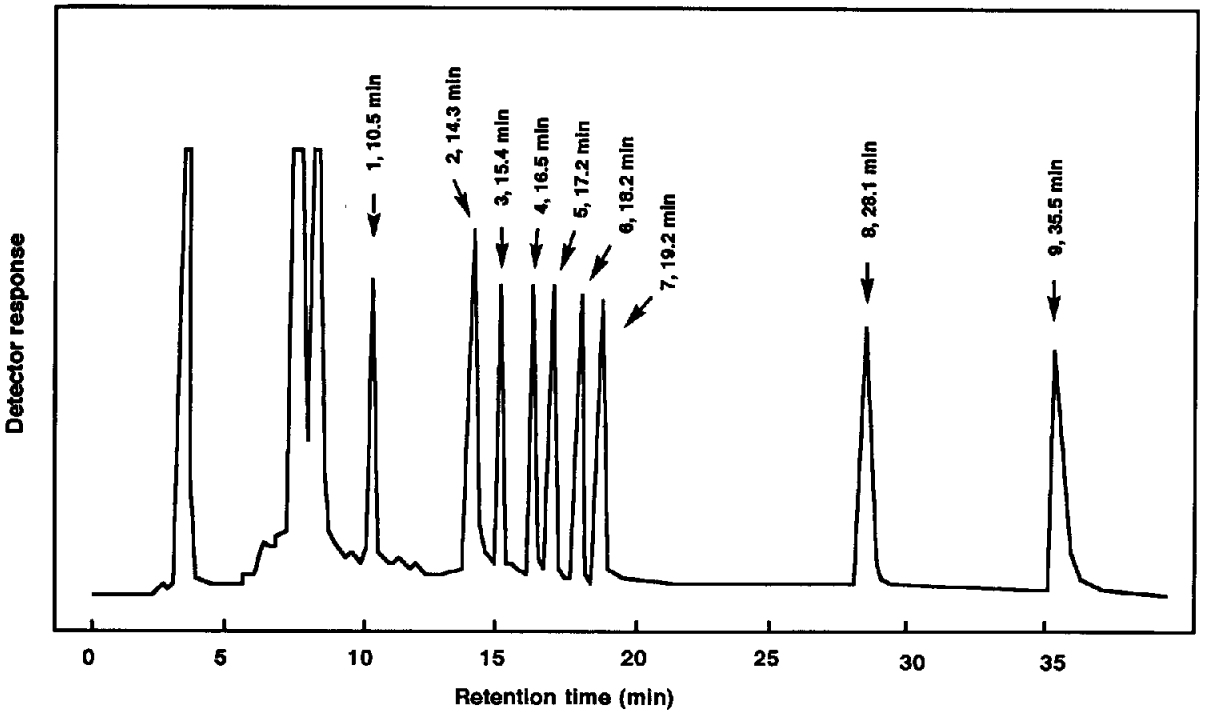

Fig. 4. Chromatogram of CDB derivatives. Concentration of estrogen used = 100 ng/ml. Peaks: 1 = estriol; 2 = *p*-hydroxybenzoic acid *sec.*-butyl ester (50 ng/ml, I.S.); 3 = ethynylestradiol; 4 = equilin + equilenin (50 ng/ml); 5 = estrone; 6 = estradiol; 7 = estetrol; 8 = 4-hydroxyestradiol; 9 = 2-hydroxyestradiol.

TABLE I

RECOVERY TEST ON THE APPLICATION TO THE DETERMINATION OF ESTROGENS

A 100-μl aliquot of plasma was spiked at the indicated concentrations. Average values were obtained from six runs.

Estrogen	Expected concentration (ng/ml)	Found concentration (mean ± S.D.) (ng/ml)
Estrone	10.0	9.3 ± 0.9
Estradiol	10.0	9.6 ± 0.9
Estriol	10.0	9.0 ± 1.1
Estetrol	10.0	9.2 ± 1.3
Ethynylestradiol	10.0	10.4 ± 1.0
2-Hydroxyestradiol	10.0	9.6 ± 1.3
4-Hydroxyestradiol	10.0	9.2 ± 1.2

isolation reaction product. The conversion rate was 22%.

Application to the determination of estrogens in plasma

Estrogens (estrone, estradiol, estriol, estetrol, 2-hydroxyestradiol, 4-hydroxyestradiol, ethynylestradiol, equilin and equilenin) were monitored for cancer [5–9] or bone diseases [10–12]. Immunoassays, receptor-binding assays [1,2,5–8,10–12], GC–MS [17,18] and HPLC with photometric detection [3,4,9,13,14] or ED [15] and with pre-column fluorimetric [19] and chemiluminescence [20] derivatization have been used widely. Sensitive determination methods are useful for monitoring of trace amounts of estrogens in blood and urine. We therefore applied the proposed CDB method to the determination of estrogens in plasma.

Recovery test. The recovery of 1.0 ng (10 ng/ml) of estrogens added to 100 μl of plasma is shown in Table I. Average recoveries (n = 6) were 94.7%.

Calibration curves. Detection limits were 0.1–0.2 pg per 100 μl of plasma (signal-to-noise ratio = 3). The calibration graphs were linear in the range 2000–3000 ng per 100 μl. The within-day relative standard deviations (n = 6) for each estrogen (1.0 ng per 100 μl of plasma) were 8.7–13.0%, and day-to-day relative standard deviations (n = 6) were 9.0–12.5%. The proposed HPLC method is more than 100 times more sensitive than other HPLC methods with photometric detection [3,4,9,13,14] or ED [15] or HPLC with fluorimetric derivatization by dansyl chloride [19], and 10 times more sensitive than HPLC with chemiluminescence pre-column derivatization by dansyl chloride [20]. The application of the proposed method to monitoring of estrogens in plasma in pregnancy is in progress.

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