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Discriminating the endogenous and exogenous urinary estrogens in human by isotopic ratio mass spectrometry and its potential clinical value

Sheng Yang [a,*], Dapeng Zhang [b], Youxuan Xu [a], Xiaobing Wang [a], Xin Liu [a], Shan Wang [a], Jingzhu Wang [a], Moutian Wu [a], Zhenwen He [a], Jian Zhao [a], Hong Yuan [a]

[a] National Laboratory of Anti-Doping, China Anti-Doping Agency, Beijing 100029, China
[b] Neixiang Center for Disease Control and Prevention, Henan Province 474350, China

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ABSTRACT

Estrogens were prohibited in the food producing animals by European Union (96/22/EC directive) and added to the Report on Carcinogens in United States since 2002. Due to very low concentration in serum or urine (~pg/mL), the method of control its abuse had not been fully developed.

The endogenous estrogens were separated from urines of 18 adult men and women. The exogenous estrogens were chemical reference standards and over the counter preparations. Two patients of dysfunctional uterine bleeding (DUB) administered exogenous estradiol and the urines were collected for 72 h. The urinary estrogens were separated by high-performance liquid chromatography (HPLC) and confirmed. The exogenous and exogenous estrogens were analyzed by gas chromatography combustion isotope ratio mass spectrometry (GC–C–IRMS) to determine the $^{13}C/^{12}C$ ratio ($\delta^{13}C‰$).

The $\delta^{13}C‰$ values of reference standard of E1, E2, and E3 were -29.36 ± 0.72, -27.98 ± 0.35, -27.62 ± 0.51, respectively. The $\delta^{13}C‰$ values of the endogenous E1, E2, and E3 were -21.62 ± 1.07, -22.14 ± 0.98, and -21.88 ± 1.16, with $P < 0.01$ (t-test). Two DUB patients' urinary estradiol $\delta^{13}C‰$ values was depleted to -28.02 ± 0.33 after the administration. The progesterone, 17α-hydroxyprogesterone, pregnanediol, as well as desogestrel and ethinylestradiol from contraceptives were also determined.

Stable carbon isotope analysis can distinguish the endogenous and exogenous urinary estrogen in human.

1. Introduction

Estrogens are naturally synthesized in the ovaries, adrenal glands, and metabolized in the liver in human. The three major natural estrogens are estrone (3-hydroxyestra-1,3,5(10)-trien-17-one, E1), 17β-estradiol (estra-1,3,5(l0)-triene-3,17β-diol, E2), and estriol (estra-1,3,5(l0)-triene-3,16α,l7β-triol, E3), respectively, Fig. 1 [1,2]. Estrogens play an important role in the estrous cycle [3]. In female, estrogen levels vary through the menstrual cycle [4], with levels highest just before ovulation. They promote the development of female secondary sexual characteristics, such as breasts. In male, estrogen regulates the maturation of sperm [5]. Estradiol and estrone can be converted from testosterone and androstenedione; the conversion is catalyzed by enzyme aromatase (EC 1.14.14.1) [6,7].

Due to their important physiological effects, estrogens are strictly prescribed to the treatment of dysfunctional uterine bleeding (DUB) [8,9], menopause, and in the hormone replacement therapy (HRT) [10] in gynecological clinics. However, as a major component in the contraceptive pills, estrogens can be easily obtained over the counter. According to an epidemiological survey in Fujian province (34.8 million population), southeast China, there were 78% children ($n = 80$) diagnosed of precocious puberty had a history exposure to exogenous sexual hormone [11]. The gonadotropin-releasing hormone (GnRH) stimulating trial had to be performed to clarify the etiology, but sometimes it was refused by

Abbreviations: A, androsterone; amu, atomic units; CIR, carbon isotopic ratio; C.V.%, coefficient of variance; DUB, dysfunctional uterine bleeding; E1, estrone; E2, 17β-estradiol; E3, estriol; Etio, etiocholanolone; eV, electron volt; GC–C–IRMS, gas chromatography combustion isotope ratio mass spectrometry; GC–MS, gas chromatography mass spectrometry; GnRH, gonadotropin-releasing hormone; HCG, human chorionic gonadotropin; Hgb, hemoglobin; HPLC, high-performance liquid chromatography; HRT, hormone replacement therapy; ^{125}I, iodine element (125); I.D., inner diameter; IRMS, isotopic ratio mass spectrometry; LC–MS, liquid chromatography mass spectrometry; LOD, limit of detection; m/z, mass to charge ratio; MTBE, methyl tert-butyl ether; MSTFA, N-Methyl-N-(trimethylsilyl) trifluoroacetamide; NIEHS, National Institute of Environmental Health Sciences; PD, pregnanediol; QC, quality control; RIA, radioimmunoassay; SD, standard deviation; SPSS, Statistical Package for the Social Sciences; VPDB, Vienna Pee Dee Belemnite; WADA, World Anti-Doping Agency.

* Corresponding author. Address: China Anti-Doping Agency, No. 1, Anding Road, Chaoyang District, Beijing 100029, China. Tel.: +86 10 64946933; fax: +86 10 64979303.

E-mail address: yang_sound@sina.com (S. Yang).

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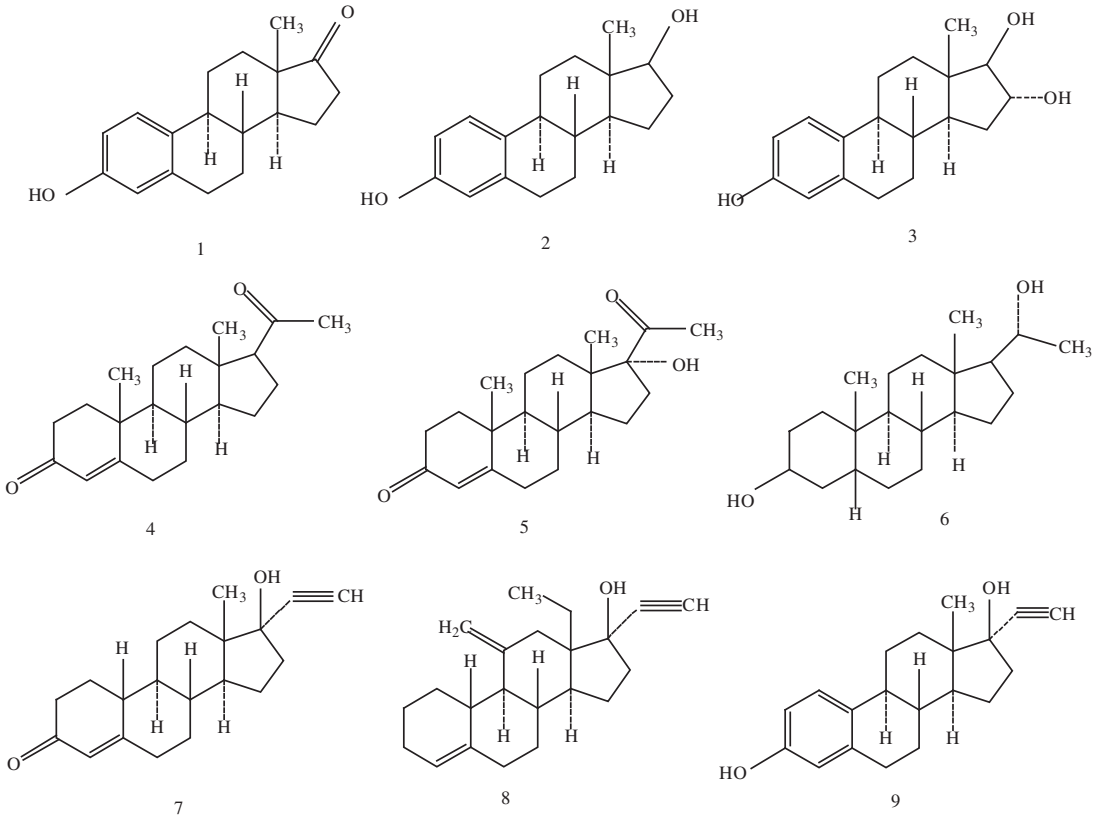

Fig. 1. The structure of estrone, 1; estradiol, 2; 17β-estriol, 3; progesterone, 4; 17α-hydroxyprogesterone, 5; pregnanediol, 6; norethisterone, 7; desogestrel, 8; ethinylestradiol, 9.

parents for the considerations of worsen the condition. If the urinary estrogens can be detected and indicated the sources, namely synthesized within the organism (endogenous) or from the outside (exogenous), the differential diagnosis would be relative safer and easier. In oncology, about 75% of breast cancers are known as hormone-sensitive or estrogen-receptor-positive cancers [12]. In European Union, anabolic agents including estrogen were prohibited in food producing animals (96/22/EC directive). And in the United States, estrogen had been added to the Report on Carcinogens by National Institute of Environmental Health Sciences (NIEHS) since the year 2002 [13]. If the preventive measures can be performed in the food safety area, nutritional supplements products, and cosmetics as well, the incidence of breast cancer may be lowered. Therefore, it is necessary to develop a method to control the estrogens abuse.

The earlier published method for quantifying estrogens had used radioimmunoassay (RIA) [14]. This technique had the limitations that radiological ^{125}I was used as a tracer, and the antibody had cross-reactivity with other substances. Although estrogens have been determined at trace levels (ng/L) by mass spectrometric methods in human serum [15], river water [16], and sewage sediments [17]; these results could not provide sufficient information about the origins of estrogens. The isotopic ratio mass spectrometry (IRMS) has been introduced in the anti-doping fields to control testosterone abuse [18], an endogenous steroid. And one publication reported the findings in cattle after administration of exogenous estrogen by this technique [19]. We hypothesized the stable carbon isotopic ratio (CIR) is different between endogenous and exogenous estrogens and asked whether this difference can be distinguished by the IRMS methods. Our study investigated the carbon isotopic ratios of chemical reference estrogens and the endogenous estrogens from 8 healthy subjects, as well as two cases of estrogen administration (DUB) using the developed methods.

2. Experimental

2.1. Chemicals

Estrone (3-hydroxyestra-1,3,5(10)-trien-17-one), estradiol (estra-1,3,5(l0)-triene-3,17β-diol), estriol (estra-1,3,5(l0)-triene-3,16α,l7β-triol), progesterone (pregn-4-ene-3,20-dione), 17α-hydroxyprogesterone (4-pregnen-17α-ol-3,20-dione), and pregnanediol (5β-pregnane-3α,20α-diol) were chemical references from Sigma–Aldrich. The 19-norethisterone (17α-ethynyl-17β-hydroxy-19-nor-4-androsten-3-one) was obtained from national institutes for food and drug control of China. The contraceptive drugs of Yasmin®, Marvelon®, and Mercilon® were purchased from the pharmacy in Beijing. Testosterone and androsterone obtained from Sigma–Aldrich, which were used as external standards for HPLC and GC–C–IRMS analysis. Methyl tert-butyl ether (MTBE) and acetonitrile were of HPLC grade obtained from Dima. Analytical grade of hydrochloric acid, sodium dihydrogen phosphate monohydrate, and disodium hydrogen phosphate dehydrate were purchased from Sinopharm Chemical Reagent. The N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol were from Sigma–Aldrich. The β-glucuronidase from *Escherichia coli* (G7396) and sulfatase from *Helix pomatia* (S9626) were obtained from Sigma–Aldrich. Pure water was made by a Milli-Q system (Millipore).

2.2. Individuals providing urine for estrogens isolation

Urine samples were collected from 18 healthy adults, including 8 females (19–21 years old) and 8 males (26–45 years old) as well as 2 pregnant women. For females, urines were collected during the menstrual cycle, from the 9th day to the 15th day, for a phys-