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Stable isotope labeling – Liquid chromatography/mass spectrometry for quantitative analysis of androgenic and progestagenic steroids

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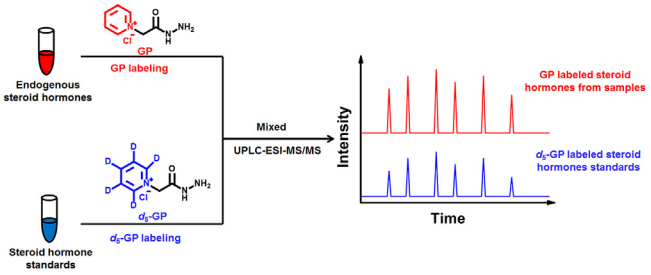
HIGHLIGHTS

- Girard reagent P (GP) and d_5-GP were synthesized and utilized to label steroid hormones.
- The ionization efficiencies of steroid hormones were greatly improved by 4–504 folds after labeling.
- d_5-GP labeled standards were used as the internal standards for quantification.
- The steroid hormones in human follicular fluid we successfully quantified.

GRAPHICAL ABSTRACT



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ABSTRACT

Steroid hormones play important roles in mammal at very low concentrations and are associated with numerous endocrinology and oncology diseases. Therefore, quantitative analysis of steroid hormones can provide crucial information for uncovering underlying mechanisms of steroid hormones related diseases. In the current study, we developed a sensitive method for the detection of steroid hormones (progesterone, dehydroepiandrosterone, testosterone, pregnenolone, 17-hydroxyprogesterone, androstenedione and 17α-hydroxypregnenolone) in body fluids by stable isotope labeling coupled with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis. In this respect, a pair of isotopes labeling reagents, Girard reagent P (GP) and d_5-Girard reagent P (d_5-GP), were synthesized and utilized to label steroid hormones in follicular fluid samples and steroid hormone standards, respectively. The heavy labeled standards were used as internal standards for quantification to minimize quantitation deviation in MS analysis due to the matrix and ion suppression effects. The ionization efficiencies of steroid hormones were greatly improved by 4–504 folds through the introduction of a permanent charged moiety of quaternary ammonium from GP. Using the developed method, we successfully quantified steroid hormones in human follicular fluid. We found that the contents of testosterone and androstenedione exhibited significant increase while the content of pregnenolone had significant decrease in follicular fluid of polycystic ovarian syndrome (PCOS) patients compared with healthy controls, indicating that these steroid hormones with significant change may contribute to the pathogenesis of PCOS. Taken together, the developed stable isotope labeling coupled LC-ESI-MS/MS analysis demonstrated to be a promising method for the sensitive and accurate determination of steroid hormones, which may facilitate the in-depth investigation of steroid hormones related diseases.

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1. Introduction

Steroid hormones that are developed from cholesterol in adrenal cortex, gonads and placenta play important roles in mammal at very low concentrations [1]. It has been reported that steroid hormones are associated with numerous endocrinology and oncology diseases, including congenital adrenal hyperplasia [2], adrenal insufficiency [3], chronic pelvic pain [4], prostatitis and oncology (breast cancer) [5,6]. And testosterone is normally used to estimate hypogonadism in male as well as to evaluate hyperandrogenism, hirsutism and infertility in female [7]. Excessive production of androstenedione is related with tumors of ovarian and andrenol origin, polycystic ovarian syndrome [7]. Thus, accurate measurement of steroid hormones is necessary for the diagnosis and treatment of diseases related with the disorders of steroid hormone synthesis and metabolism.

Various methods have been developed to measure steroid hormones, including immunoassay [8], gas chromatography/mass spectrometry (GC/MS) [9], and high performance liquid chromatography/mass spectrometry (LC/MS) analysis [10]. Immunoassay, a traditional method for steroid hormones analysis, is relatively simple; but immunoassay easily leads to cross reactivity and the specificity and sensitivity are normally poor. In addition, multiple steroid hormones can't be simultaneously determinated by immunoassay [7]. In 1963, GC/MS was first used to analyze steroid hormones [11]. Although GC/MS can distinctly improve the specificity for the measurements of steroid hormones, the detection sensitivity is limited, and the sample preparation is time-consuming [12]. LC/MS is frequently employed for the analysis of steroid hormones because of its high specificity and sensitivity [12–14]. However, the ionization efficiencies of steroid hormones under positive ion mode are poor in electrospray ionization; therefore, the low detection sensitivity is a major problem in the analysis of steroid hormones. In addition, matrix effects often occur in the measurement of low abundant steroid hormones using LC/MS, which may lead to larger quantitation deviation in the absence of stable isotope labeled steroid hormones as internal standards.

To resolve these problems, stable isotope labeled steroid hormones can be used as internal standards to overcome the matrix effects and ionization differences for the accurate quantification. However, few stable isotopes labeled steroid hormone is commercially available. In this respect, in vitro stable isotope labeling strategy has been developed for quantitative profiling of metabolites [15]. The typical method of differential isotope labeling normally uses chemical labeling to introduce a light isotope tag to the target compounds in sample and a heavy isotope tag to standards, followed by mixing the light labeled sample and heavy labeled standards for MS analysis. The heavy labeled standards can be used as the internal standards to minimize the quantitation deviation due to matrix and ion suppression effects. In addition, chemical labeling also can improve the ionization efficiencies of target compounds through introduction of permanently charged moieties or easily protonated moieties [16–19]. Recently, our group has also used the stable isotope labeling strategy coupled with LC/MS analysis for the determination of phytohormones, carboxyl- and thiol-containing metabolites in biological samples [20–23].

In this work, we developed stable isotope labeling strategy combined with LC/MS analysis for the accurate and sensitive detection of seven steroid hormones in the biosynthetic pathway from cholesterol to testosterone (Fig. 1) [24]. In this respect, a pair of isotopes labeling reagents, Girard reagent P (GP) and d_5-Girard reagent P (d_5-GP), were synthesized and used to label steroid hormones in sample and steroid hormone standards, respectively. And then the light and heavy labeled samples were combined for LC/MS analysis. The heavy labeled steroid standards were used as the internal standards to minimize the quantitation deviation due to the matrix and ion suppression effects in MS analysis. In addition, the introduction of a permanent charged moiety of quaternary ammonium of GP into the steroid hormones dramatically enhanced the detection sensitivities by 4–504 folds. With the developed method, we analyzed the steroid hormones in follicular fluid of polycystic ovarian syndrome (PCOS) patients that is one of the most common endocrine disorders affecting 5–7% of women of reproductive age [25].

2. Experimental

2.1. Chemicals and materials

Dehydroepiandrosterone, testosterone, pregnenolone, 17-hydroxyprogesterone, ethyl chloracetate were purchased from Energy Chemical Co., Ltd (Shanghai, China). Progesterone and androstenedione were purchased from Aladdin Industrial Co., Ltd (Shanghai, China). 17α-hydroxypregnenolone was purchased from J&K Co., Ltd (Beijing, China). The structures of these steroid hormones are shown in Fig. 1 d_5-pyridine was purchased from Sigma (St. Louis, MO, USA). Analytical grade pyridine, ethyl alcohol, hydrazine hydrate, methyl tert-butyl ether (MTBE), formic acid and glacial acetic acid (AcOH) were supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). HPLC-grade acetonitrile (ACN) and methanol (MeOH) were obtained from TEDIA Co. Inc. (Ohio, USA). Ultrapure water was purified by a Milli-Q apparatus (Millipore, Bedford, MA).

The stock solutions of GP (20 μmol/mL), d_5-GP (20 μmol/mL) were prepared in HPLC-grade MeOH. The stock solutions of steroid hormone standards were prepared in HPLC-grade MeOH at the concentration of 1 μM for each. All stock solutions were stored at −20 °C and avoid light.

2.2. Synthesis and characterization of GP and d_5-GP

GP was synthesized according to previously reported method [26]. And the procedure for the synthesis of GP and d_5-GP are shown in Figure S1 in Supporting Information. Briefly, ethanol (12 mL), ethyl chloroacetate (6.13 g, 50 mmol), and anhydrous pyridine (3.96 g, 50 mmol) were add to a 100-ml round-bottom flask successively and the mixture was refluxed for 6 h. The resulting mixture was cooled to room temperature; then the hydrazine hydrate (2.5 g, 50 mmol) was added with shaking. The product was precipitated after 30 min. The solid product was filtered out, washed with cold anhydrous ethanol, and dried in vacuum drying oven. Finally the GP was obtained (yield, 71.9%). The isotope labeling reagent of d_5-GP was synthesized using the same procedure except that d_5-pyridine was used instead of pyridine (Figure S1, Supporting Information).

2.3. Sample preparation

The follicular fluid samples from PCOS patients (n = 11) and healthy control people (n = 11) were collected from Renmin Hospital of Wuhan University (Wuhan, China). Healthy control follicular fluid samples were selected based on medical history and physical examination. The collected samples were stored at −80 °C until use. All the experiments were performed in accordance with Renmin Hospital of Wuhan University Ethics Committee's guidelines and regulations.

The extraction of steroid hormones from the follicular fluid samples was carried out according to previously reported method [27]. Briefly, the follicular fluid samples were thawed at 4 °C. And then 1 ml of extraction solvent (MTBE) were added to a