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Review

Methodological approach to the intracrine study and estimation of DHEA and DHEA-S using liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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ABSTRACT

A reliable and sensitive method for analyzing steroids using liquid chromatography tandem mass spectrometry (LC–MS/MS) is required for research concerning dehydroepiandrosterone (DHEA), which plays a central role in steroid hormone biosynthesis and metabolism. Furthermore, after the first proposal of the concept of intracrine DHEA, stable isotope tracer analysis, which is useful for structural recognition as well as determination of steroids, has been required to evaluate physiological action and hormone biosynthesis/metabolism in target organs. We describe sample processing and analysis methods for simultaneous quantification of multiple hormones, including DHEA, in serum, saliva and tissue using LC–MS/MS. A derivatization technique compatible with each functional group for measuring 3β-hydroxy-5-enes, such as DHEA and 5α/5β-steroids, with high sensitivity and specificity is also described. Finally, we describe a newly developed method for intracrine research using stable isotope-labeled ^{13}C-steroid substrates with tracer analysis of their metabolites by LC–MS/MS.This article is part of a Special Issue entitled'Essential role of DHEA'.

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1. Introduction

In this section, we describe a new method of intracrine research utilizing the latest ultra-sensitive analytical technique for steroid hormones of liquid chromatography–tandem mass spectrometry (LC–MS/MS), with a focus on dehydroepiandrosterone (DHEA). DHEA and its sulfated form (DHEA-S) are the most common serum steroids and are mostly produced by the adrenal zona reticularis in humans. Although DHEA is known to decrease with age and has been thought of merely as an intermediate product that is metabolized into active estrogens or androgens, new physiological actions of DHEA have recently received attention, since it is a precursor of androgens [1,2]. However, it is difficult to determine the physiological actions of DHEA in animal models, such as dogs and rodents, since serum DHEA (or DHEA-S) is produced not by the adrenal glands but by the gonads, making it impossible to exclude the effect of testosterone from physiological androgenic actions in animal models [3]. Thus, human samples are required for studies on the physiological actions of DHEA in humans. Moreover, a precise method for the simultaneous quantitation of several steroids in a small amount of sample is also essential for this physiological research.

LC–MS/MS can easily separate quantitative targets by high-performance liquid chromatography (HPLC) and can measure even heat-labile, non-volatile substances or isomeric steroidal compounds that are difficult to measure by gas chromatography. By utilizing the product ions derived from molecular ions for target substances and stable isotope-labeled internal standards, LC–MS/MS can simultaneously measure targets and their chemical derivatives with high specificity and sensitivity. Thus, LC–MS/MS has an advantage over immunoassay in terms of accuracy and precision in the measurement of steroid levels in basic research fields using blood, tissue (organ, hair or nail), saliva or tissue culture samples [4–8]. Many clinical researchers also have indicated the advantage of LC–MS/MS in the clinic for the measurement of very low levels of steroids, such as estradiol, testosterone and aldosterone, which often show higher values in traditional immunoassays [9–11]. Hence, LC–MS/MS analysis has become the standard method for measuring steroids in the clinical field and is utilized not only for the medical treatment of endocrine diseases, but also for the study of psychogenic depression or adverse effects of endocrine disruptors [7,12–14].

In recent years, steroidogenesis and metabolism in target organs have been the center of attention in clinical research. Labrie et al. proposed the mechanism of local production of physiologically active hormones in target organs as “intracrinology” [15]. Target organs of DHEA and their normal functions (e.g., the brain and memory, the skin and blood flow) are being studied intracrinologically [16,17]. The intracrinological relationship between DHEA and disease progression is mostly studied in breast cancer tissues, where the conversion of DHEA to estrogen via androstenedione contributes to disease progression [18]. Although radioactive steroids have been used for intracrine research, there have been several problems, such as difficulties in purifying and identifying the radioactive substances because of their low levels. Therefore, we describe a newly developed method for intracrine research using stable isotope-labeled ^{13}C-steroid substrates and tracer analysis of their metabolites by LC–MS/MS.

2. Ionization and derivatization

2.1. Methods of ionization

The main ionization methods used for LC–MS/MS analysis of steroids are atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI) and electrospray ionization (ESI). Of these three ionization methods, ESI is easily the most sensitive for polar steroids and derivative steroids [19].

Although most steroids, including DHEA, are easy to ionize by positive-mode ESI after acetonitrile-methanol-based HPLC, several steroids such as steroid sulfates (including DHEA-S and estrogen sulfate), estrogen and aldosterone, are also ionized by negative-mode ESI after HPLC [11,20]. These negatively ionized steroids tend to have higher log P values and acidic groups within their structures, and the response is observed to be generally higher when methanol rather than acetonitrile is used as the elution solvent [21]. The negative ion mode in LC–MS/MS was recently used for steroid determination in organism samples due to its ability to reduce the background matrix level. Moreover, the recent development of mass spectrometers with high-speed switching between positive and negative ion modes enabled simultaneous determination of both steroids and their sulfates [20].

APCI is used for steroids that are difficult to ionize by ESI. Moreover, APPI, which was recently developed as an alternative to APCI, enables the mass to charge ratios of samples to be analyzed more sensitively, compared with APCI [22].

2.2. Chemical structures

Steroids are divided into to five skeletal types—estrane (C18), androstane (C19), pregnane (C21), cholane (C24) and cholestane (C27)—according to the number of C-atoms. Characteristic structural differences, such as the position of double bonds, carbonyl group and hydroxyl groups, influence the ionization efficiencies of these steroids. For instance, the ionization efficiencies of 3-oxo-4-ene steroids, such as androstenedione, progesterone and corticoid, are high because they possess a 3-oxo-4-ene structure to which a proton is easily added by ESI. On the other hand, the ionization efficiencies of 3β-hydroxy-5-ene steroids, such as DHEA and pregnenolone, and of 5α/5β androstane and pregnane are low because of their low proton affinities, resulting in poor responses using ESI or APCI. The sensitivity of estimation for these steroids is low, with the lower limit of the estimation range being 50–100 pg [23,24].

2.3. Derivatization

To improve sensitivity, derivatization of the functional groups of target steroids was achieved by adding a moiety with proton affinity to the target steroids using reagents, as shown in Table 1 [25]. The hydroxyl groups of DHEA or DHT are reacted with picolinic acid (Fig. 1a) or fusaric acid (Fig. 1b), and the ester derivative obtained gives high sensitivity [26,27]. The reagent pyridinium (Fig. 1c) gives a stable ether derivative [28]. Specifically, the hydroxyl groups in the structures of several steroids, including DHEA (position 3), are likely to react with reagents with electron-donating groups, such as functional nitrogen and acidic groups (Fig. 1).

The carbonyl groups of DHEA, pregnenolone, DHT or 4-ene-3-one steroids are reacted with 2-hydrazino-1-methylpiridine (HMP) to give imino derivatives [23,29,30]. The other reagents are used for derivatization [31].

These hydroxyl and carbonyl group derivatives are very effective in achieving the high sensitivity required to detect 5-ene steroids and 5α/5β-reduced steroids. A 20–200-fold improvement in sensitivity was achieved for the target steroid DHEA. However, most reagents, including HMP and hydroxylamine, form two isomeric (*cis* and *trans*) products with 3-oxo-steroid. The chromatographic separation of DHEA derivatives from these isomers not only complicates quantitative analysis but also increases the susceptibility to interference by endogenous compounds.