Profiling of urinary steroids by lithium ion adduction-based UPLC-MS/MS

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Abstract

RATIONALE: Urine contains free and conjugated steroids. Total and free steroids were comprehensively quantified; UPLC-MS/MS based on Li adduction allowed for detecting thirteen 3-OH-containing steroids, two of which were detected in human urine for the first time. **METHODS:** Free urinary steroids were isolated by solid-phase extraction (SPE) with 80% acetonitrile. The total steroids were prepared by enzymatic treatment of urine with a cocktail of sulfatase and glucronidase, protein precipitation, and separation with the above SPE. The free and total steroids were separately analyzed by UPLC-MS/MS with and without introduction of Li^+ solution. The steroids were quantified by two standard curves created using product ion transitions derived from MH^+ and $[M+Li]^+$. **RESULTS:** Two groups of human urine, male and female urine, were analyzed. The absolute amount of each steroid was determined based on creatinine levels. The differences between the male and female groups are clearly attributable to sex steroids. 7-OH P5 and 7-OH DHEA were, for the first time, quantified in the total steroids of female urine, and the latter was identified in both female and male urine. **CONCLUSIONS:** By combining UPLC-MS/MS based on lithium ion incorporation with conventional UPLC-MS/MS, a total of 29 steroids were identified in human urine containing two newly found steroids.

Profiling of urinary steroids bylithium ion adduction -based UPLC-MS/MS

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Abbreviations : 3-OH steroids, Steroids that contain a 3-hydroxyl group; 11-OH-An, 11-β-hydroxyandrosterone; 16-OH-E1, 16-hydroxyestrone; 6-OH-E1-$^{13}C_3$, 16-hydroxyestrone-2,3,4-$^{13}C_3$; 17-OH-P4, 17α-hydroxyprogesterone; 17-OH-P4-$^{13}C_3$,17α-hydroxyprogesterone-2,3,4-$^{13}C_3$; 17-OH-P5, 17α-hydroxypregnenolone; 17-OH P5-d_3, 17α-hydroxypregnenolone-21,21,21-d_3; 7-OH DHEA, 7α-hydroxydehydroepiandrosterone; 7-OH P5, 7α-hydroxypregnenolone; ACN, acetonitrile; AcOH, acetate acid; AE, androstenedione; AE-$^{13}C_3$, androstene-3,17-dione-2,3,4-$^{13}C_3$; al-Preg, allopregnenolone; An, androsterone; APD, alphadolone; AT, adrenosterone; CID, collision induced dissociation; COB, corticosterone; COB-d_4, corticosterone-9,11,11,12-d_4; COL, Cortisol; COL-d_4, cortisol-9,11,12,12-d_4; COR, cortisone; COR-$^{13}C_3$, cortisone-2,3,4-$^{13}C_3$; COS, 11-deoxycortisol; DHEA, dehydroepiandrosterone; DHEA-

d_5, dehydroepiandrosterone-2,2,3,4,4-d_5; DHT, dehydrotestosterone; DOC, 11-deoxycorticosterone; E1, estrone; E1-$^{13}C_3$, estrone-2,3,4-$^{13}C_3$; E2, estradiol; E3, estriol; E3-d_3, estriol-d_3; ESI, electrospray ionization; LiCl, lithium chloride; MeOH, methanol; MRM multiple reaction monitoring, P4, progesterone; P4-$^{13}C_3$, progesterone-2,3,4-$^{13}C_3$; P5, pregnenolone; P5-$^{13}C_2$,d_2, pregnenolone-$^{13}C_2$,d_2; SPE, solid-phase-extraction; TE, testosterone; TE-$^{13}C_3$,testosterone-2,3,4-$^{13}C_3$; THB, tetrahydrocorticosterone; TH-COL, tetrahydrocortisol; TH-COR, tetrahydrocortisone; TH-COR-d_6, tetrahydrocortisone-2,2,4,4,21,21-d_6-d_6; TH-DOC, 3β,5α-tetrahydrodeoxycorticosterone; THS, tetrahydrodeoxycortisol.

Running title: Steroid profiling of human urine

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METHODS: Free urinary steroids were isolated by solid-phase extraction (SPE) with 80% acetonitrile. The total steroids were prepared by enzymatic treatment of urine with a cocktail of sulfatase and glucronidase, protein precipitation, and separation with the above SPE. The free and total steroids were separately analyzed by UPLC-MS/MS with and without introduction of Li^+ solution. The steroids were quantified by two standard curves created using product ion transitions derived from MH^+ and $[M+Li]^+$.

RESULTS: Two groups of human urine, male and female urine, were analyzed. The absolute amount of each steroid was determined based on creatinine levels. The differences between the male and female groups are clearly attributable to sex steroids. 7-OH P5 and 7-OH DHEA were, for the first time, quantified in the total steroids of female urine, and the latter was identified in both female and male urine.

CONCLUSIONS: By combining UPLC-MS/MS based on lithium ion incorporation with conventional UPLC-MS/MS, a total of 29 steroids were identified in human urine containing two newly found steroids.

Keywords: human urine, conjugated steroids, Li adduction, UPLC-MS/MS

Introduction

Steroids play important roles in various physiological functions of the organism, including brain development, behavior, cognition, neuroplasticity, and neuroinflammation, and are metabolized and excreted in the urine as conjugated forms[1-4]. Transient concentrations of each steroid in the blood indicate a physiological state, but these concentrations change rapidly in response to various physiological and even psychological changes[5,6].

Urinary steroids represent the total amount of each steroid circulating in the blood over a given time period. Profiling of urinary steroids should be important for diagnosing relatively long-term physiological changes such as chronic disease, breast cancer and prostate cancer[1,7,8]. Steroid hormones are enzymatically synthesized from cholesterol and classified into various types of steroids, including estrogens (female reproductive steroids), androgens (male reproductive steroids), progestogens (pregnancy steroids), and corticosteroids (stress steroids)[2,4]. After circulating in the blood and functioning in their respective organs, these steroids are enzymatically converted to inactive substances linked with sulfate or glucuronic acid in several organs such as liver and prostate, and excreted in urine[4,7,9]. Due to their diverse structures, these conjugated steroids are usually converted back to free steroids for the analysis; The advantage of urine is that it is non-invasive, therefore it has no pain and is easy to collect in large amounts[10]. LC/ESI-MS/MS has become a popular method for steroid analysis in biological samples due to its high sensitivity, high selectivity and the ability to run multiple analytes simultaneously[11-13]. However, 26 of the 29 steroids contain hydroxyl groups in their backbone, which are prone to dehydration when ionized by positive-ion ESI. Such dehydration diversifies the precursor ion, thereby reducing sensitivity and hindering their quantification by MRM methods. To overcome this issue, we recently reported a method to protect steroid molecules from dehydration by using Li+ as the adduct ion to steroid ("Li method")[14].

Application of the Li method to the analysis of human urine resulted in the detection of 13 3-OH steroids with 1.3-20 times greater sensitivity than conventional LC/ESI-MS/MS ("H method"). Other steroids (16 species) were identified by the H method. As a result, a total of 29 steroids were identified.

Materials and Methods:

Chemicals

HPLC grade methanol, acetonitrile, 99.998% trace metals basis lithium chloride, analytical-reagent-grade acetate acid, L-Ascorbic acid, β-glucuronidase-arylsulfatase from Helix pomatia, sodium bicarbonate, corticosterone, cortisol, cortisone, dehydroepiandrosterone, dehydrotestosterone , 11-deoxycorticosterone, 11-deoxycortisol, estriol, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, pregnenolone, progesterone, testosterone, androstene-3,17-dione-2,3,4-$^{13}C_3$, corticosterone-9,11,11,12-d_4, cortisol-9,11,12,12-d_4, cortisone-2,3,4-$^{13}C_3$, dehydroepiandrosterone-2,2,3,4,4-d_5, estrone-2,3,4-$^{13}C_3$, 17α-hydroxypregnenolone-21,21,21-d_3, 17α-hydroxyprogesterone-2,3,4-$^{13}C_3$, pregnenolone-20,21-$^{13}C_2$-16,16-$d_2$solution, progesterone-2,3,4-$^{13}C_3$ and testosterone-2,3,4-$^{13}C_3$ were purchased from Sigma-Aldrich (Tokyo, Japan). Alphadolone , allopregnenolone , tetrahydrocortisol, tetrahydrocortisone, 11-β-hydroxyandrosterone, tetrahydrocorticosterone, tetrahydrodeoxycorticosterone, tetrahydrodeoxycortisol, 16-hydroxyestrone, 16-hydroxyestrone-2,3,4-$^{13}C_3$, estriol-d_3 and tetrahydrocortisone-$d_6$were supplied by Toronto Research Chemicals (North York, Canada). Adrenosterone, androsterone, androstenedione, estrone, and estradiol was purchased from Tokyo Chemical Industry (Tokyo, Japan). 7α-hydroxydehydroepiandrosterone and 7α-hydroxypregnenolone were supplied by Nacalai tesque (Kyoto, Japan). HPLC grade formic acid was obtained from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). Ultrapure water was prepared using a Organo puric ω (Tokyo, Japan). The HF Bond Elute C18 (1 mL, 50 mg) was purchased from Agilent Technologies, Inc. (Santa Clara, CA). Creatinine (urinary) Colorimetric Assay kit was purchased from Cayman Chemical (Ann Arbor, MI). The amount of creatinine (mg) was measured according to the manufacturer's manual.

Human urine and ethical approval

The healthy samples of human urine (3 males, 3 females) without personally identifiable information were purchased from Innovative Research, Inc. (Novi, MI). This study was approved by the Ethics Committee of the Institute for Protein Research, Osaka University (No. 2020-3). Human studies abided by the Declaration of Helsinki principles.

Sample preparation

Steroid extraction strategy should be developed, taking into consideration nature of the matrix, cleaning and pre-concentration step, and detection of conjugated steroid[1,15,16]. 240 μL urine were first mixed with the internal standard mixture solution (2.4 uL) (see **EXPERIMENTAL SECTION** in the "**Supplemental information** "), mixed with 240 μL of freshly prepared 0.15 M sodium acetate buffer (pH 4.6) containing 1.5 mg *L* -ascorbic acid and 4 μL of β-glucuronidase-arylsulfatase solution. After the reaction, the solution was allowed to stand at 37 °C for 16 h, 960 μL of ACN was mixed in a 2 mL Eppendorf tube (Eppendorf AG, Hamburg, Germany), vortexed for 30 s and allowed to stand at 4 °C for 30 min. The mixture was centrifuged at 21,000 x g for 15 min at 4°C and the protein precipitate was spun down. The supernatant was transferred to a 15 mL Eppendorf tube and 8.16 mL of H_2O was added to give a final concentration of 10% ACN (v/v). The mixture was centrifuged at 19,000 x g for 15 min at 4°C and the supernatant was loaded on a Bond Elute C18 column (50 mg) that had been washed with 1 mL of aqueous 80% ACN twice and equilibrated with 1 mL of aqueous 10% ACN twice. Samples were washed three times with 1 mL of 10% ACN and collected with 1 mL of 80% ACN in 1.5 mL Eppendorf tubes. The eluate was evaporated to dryness using a speed-vac and the resulting residue was redissolved in 24 μL of 40% MeOH. The mixture was centrifuged at 20,000 x g for 15 min at room temperature and the supernatant was transferred to a 250 μL inactivated glass insert. The insert was placed in a vial and subjected to LC-MS/MS.

LC/ESI-MS/MS

The UPLC-MS/MS was performed according to the method described previously[14] using an Agilent 1290

Infinity II and 6470 triple quadrupole mass spectrometer equipped with an ESI ion source (Agilent Technologies, Inc. Santa Clara, CA). Briefly, the samples were separated with an Agilent Eclipse Plus C18 RRHD 2.1x100 mm, 1.8 μm column by using solvent A (0.1% formic acid in deionized water) and solvent B (0.1% formic acid in MeOH). The elution gradient was from 40-80.0 % B from 0 to 8 min, maintained at 80 % B from 8 to 10 min, 80-40.0 % from 10.0 to 10.10 min and held at 40 % B from 10.1 to 13.1 min. The injection volume was 20 μL. The MRM mode was applied for the detection and quantitation of all steroids with two transitions optimized for each targeted compound (Table S1). The post-column addition of 0.2 mM LiCl in H_2O was carried out with an Agilent 1100 binary pump as the auxiliary pump. The column effluent (0.4 mL min^{-1}) and auxiliary solution (0.4 mL min^{-1}) were mixed at the T-connector and passed through two in-line filters (Agilent 1290 inline filter, 0.3μm), which were connected in tandem, prior to reaching to the ion source (Figure S1). The auxiliary pump was off when conducting the "H method".

Data processing

Data were acquired using an Agilent MassHunter Acquisition system, and processed using Agilent MassHunter Quantitive Analysis, Microsoft Excel, and OriginPro 2018J (Academic).

Results and discussion

Free and total steroids were quantified for male and female urine. Steroids were comprehensively analyzed using the Li and H methods. As discussed in the previous paper, 3-OH steroids severely undergo dehydration when ionized in positive ion mode[14]. Furthermore, additional OH group(s) in the backbone, such as7-OH DHEA and 7-OH P5, further promote dehydration during ionization and those are difficult to detect by conventional LC/ESI-MS/MS. The adduction of Li ions to steroid molecules in the ionization could protect them from dehydration, resulting in an increase in ion intensity of the precursor ion as a lithiated form and efficient generation of specific fragment ions, especially, for 3-OH steroids[14] (Figure 2 and Figure S2). For 3-ketosteroids, the "H method" could be used because they do not contain such hydroxyl groups. Furthermore, the analytical settings of the H-method and the Li-method are very similar, so they can be operated in a consecutive manner with a regular LC-MS/MS, allowing for a more comprehensive analysis of multi-class steroids in bio-samples. Figure 2 compares MRM chromatograms of free and total 7-OH DHEA and 7-OH P5 from female and male specimens; 7-OH P5 was only observed in total steroids of female specimen, while 7-OH DHEA was observed in free and total steroids prepared from female and male samples. These two steroids were first identified by the Li method in human urine. When these steroids were measured by the H-method, 7-OH P5 was barely observed and 7-OH DHEA was likely to be observed as a shouldered peak, but the retention time and peak height ratio of the two transitions differed significantly from the standard one (Figure 2, right panel). This could be partly attributed to instability of the protonated ion, MH^+ or $(M\text{-}H_2O+H)^+$ ions, in the ion source. The adduction of a Li ion to steroid in the gas phase should protect it from the dehydration or degradation during ionization. Indeed, the sensitization ratios of 7-OH-DHEA and 7-OH-P5 were 7.1 and 11.1, respectively (Figure 2).

19 free and 29 total steroids were identified in the urine of men and women. The amounts of each steroid was normalized by creatinine values obtained separately for male and female samples (Table S5). The relative composition of free steroids was different from the total steroids, the majority of which consists of the conjugated forms. The predominant urinary steroids (conjugated forms) were TH-COL, TH-COR, APH, COB, THB, An, DHEA, and 11-OH-An. Female had relatively higher contents of 7-OH-P5, 16OH-E1, E3, P5, and 7-OH-DHEA than male, while male had higher contents of DOC, AT, TH-DOC, TE, COR, COL THB, 11-OH-An and An, a result that clearly reflects gender differences (Figure 3). Since 7-OH-P5, 16OH-E1, E3, P5, 7-OH-DHEA, AT, TE, 11-OH-An and An are sex steroids, it is normal for significant differences between male and female urine samples. The stress-related corticosteroids DOC, TH-DOC, COR, COL and THB were common in male[17], however, this result needs to be confirmed with more specimens. Note that the amounts of each steroid were obtained for the mixture of three specimens of urine and their levels were almost in the ranges of those reported previously[2,18-25] (Figure S5), which were obtained for individuals' urine or 24-hours pooled urine[22], while E2, THB were observed with higher levels than those reported previously. It could be ascribable to inter-day or individual variation of steroid metabolism.

The high sensitivity of the Li method also revealed the profile of free steroids in urine (about 1.2% of total steroid levels), although their physiological function is unclear. It is worth mentioning that since the method allowed the reliable quantification of 7-OH DHEA, which has been reported to be involved in "doping", it will be useful for its monitoring[26].

Conclusions:

Lithium ion adduction-based UPLC-MS/MS was applied to urinary steroid analysis and thirteen 3-OH steroids were detected. Among them, 7-OH P5 and 7-OH DHEA were identified for the first time in human urine. A total of 29 steroids and 19 free steroids were identified by combining conventional methods to detect protonated ions. The urinary steroid profile obtained in this study is useful for monitoring relatively long-term physiological conditions and diagnosing chronic diseases.

Figure 1. Biosynthesis map of steroid hormones.

Urinary steroids identified in this study are shown, of which 3-OH steroids are shown in green and newly identified steroids are shown in red.

Figure 2. MRM chromatograms of newly identified 7-OH P5 (left) and 7-OH DHEA (right) in human urine. Chromatograms were obtained for standard (2 ng for each, topmost), female-total (second), male-total (third), female-free (fourth), male-free (fifth) by the Li method (left) and the H method (right). The values above the peaks indicate retention times and the values in brackets indicate the peak-height ratio of the two peaks in each chromatogram observed at the two MRM transitions set for each compound (see Table S1). Typical fragmentation ions of lithiated 3-OH steroids are depicted in their structures.

Figure 3. Pie chart of relative amounts of major steroids in male (a) and female urine (b) and bar chart of free and total steroid levels (ng/creatinine (mg)) obtained in male (top) and female (bottom) urine (c). All the data were summarized in Table S5.

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CRediT Classification:

YP : Investigation, Conceptualization, Formal Analysis, Writing – Original Draft Preparation, Visualization,. **QW** : Investigation, Conceptualization, Formal Analysis. **MC** : Investigation, Review & Editing. **TT** : Conceptualization, Resources, Funding Acquisition, Supervision, Writing – Review & Editing.