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RESEARCH ARTICLE

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Profiling of urinary steroids aided by lithium ion adduction-based ultrahigh-performance liquid chromatography–tandem mass spectrometry

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Rationale: As 3-OH-containing steroids are prone to dehydration by conventional electrospray ionization, reducing detection sensitivity, Li ion adduction-based ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC/MS/MS), developed to prevent dehydration and effectively detect 3-OH steroids, was applied for profiling total and free steroids in urine.

Methods: Free urinary steroids were isolated directly from urine by solid-phase extraction (SPE) with 80% acetonitrile. The total steroids were prepared by enzymatic treatment of urine with a cocktail of sulfatase and glucuronidase, protein precipitation, and separation with the above SPE. In order to detect as many steroid types as possible, UHPLC/MS/MS (Li method) with Li^+ solution added after the column was used for analysis in addition to the conventional method of detecting protonated ions (H method). The 13 3-OH steroids and the remaining 16 steroids were quantified by standard curves prepared using product ion transitions derived from $[M + Li]^+$ and MH^+, respectively.

Results: Two groups of human urine, male and female urine, were analyzed. 3-OH steroids could be detected with greater sensitivity using the Li method than the conventional method. The absolute amounts of each steroid were normalized based on creatinine levels. The difference between the male and female groups are clearly attributable to sex steroids.

Conclusions: Twenty-nine total steroids and 19 free steroids were identified in a limited volume (240 mL) of urine. Of these, 13 3-OH steroids were better detected by Li^+ adduction-based UHPLC/MS/MS.

1 | INTRODUCTION

Steroids play important roles in various physiological functions, 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

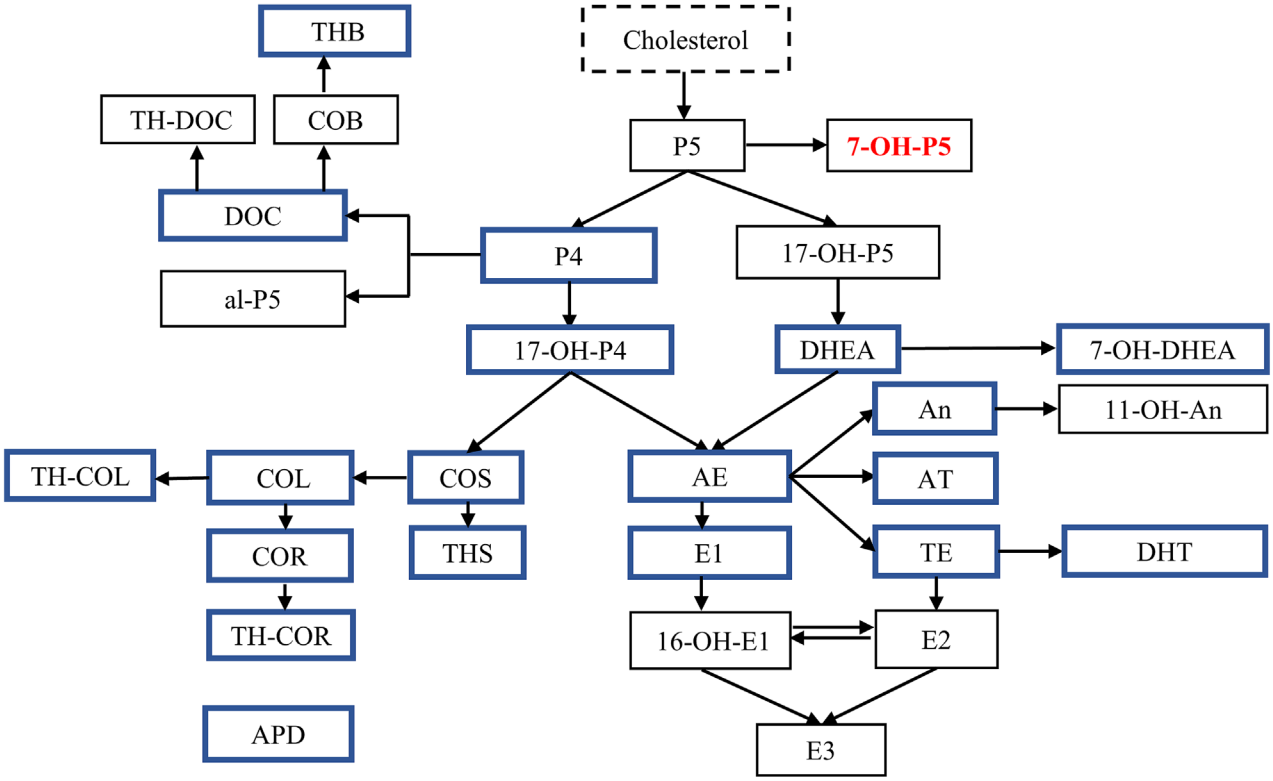

FIGURE 1 Biosynthesis map of steroid hormones. Steroids (total steroids) identified in this study are boxed with a solid line, and free steroids are boxed with a thick dark-blue line. Newly identified steroid is shown in red [Color figure can be viewed at wileyonlinelibrary.com]

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] (Figure 1). 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 analysis. The advantage of urine is that it is non-invasive; therefore it involves no pain and is easy to collect in large amounts.[10] Liquid chromatography (LC) combined with electrospray ionization (ESI) tandem mass spectrometry (MS/MS) has become a popular method for steroid analysis in biological samples due to its high sensitivity and high selectivity and the possibility of running multiple analytes simultaneously.[11–13] However, most 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 quantification by selected reaction monitoring (SRM) 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] This method allowed 3-OH steroids to be detected with higher sensitivity than conventional LC/ESI-MS/MS (H method).

In the study reported here, the Li method described above was used for the analysis of human urine in addition to the conventional method of detecting protonated ions in order to analyze as many types of steroids in human urine as possible.

2 | MATERIALS AND METHODS

2.1 | Chemicals

HPLC-grade methanol (MeOH), acetonitrile (ACN), 99.998% trace metals basis lithium chloride, analytical-reagent-grade acetate acid, L-ascorbic acid, β-glucuronidase-arylsulfatase from *Helix pomatia*, sodium bicarbonate, corticosterone (COB), cortisol (COL), cortisone (COR), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), 11-deoxycorticosterone (DOC), 11-deoxycortisol (COS), estriol (E3), 17α-hydroxypregnenolone (17-OH-P5), 17α-hydroxyprogesterone (17-OH-P4), pregnenolone (P5), progesterone (P4), testosterone (TE), androstene-3,17-dione-2,3,4-$^{13}C_3$ (AE-$^{13}C_3$), corticosterone-9,11,11,12-d_4 (COB-d_4), cortisol-9,11,12,12-d_4 (COL-d_4), cortisone-2,3,4-$^{13}C_3$ (COR-$^{13}C_3$), dehydroepiandrosterone-2,2,3,4,4-d_5 (DHEA-d_5), estrone-2,3,4-$^{13}C_3$ (E1-$^{13}C_3$), 17α-hydroxypregnenolone-21,21,21-d_3 (17-OH-P5-d_3), 17α-hydroxyprogesterone-2,3,4-$^{13}C_3$ (17-OH-P4-$^{13}C_3$), pregnenolone-$^{13}C_2$,d_2 (P5-$^{13}C_2$), d_2 solution, progesterone-2,3,4-$^{13}C_3$ (P4-$^{13}C_3$), and testosterone-2,3,4-$^{13}C_3$ (TE-$^{13}C_3$) were purchased from Sigma-Aldrich (Tokyo, Japan). Alphadolone (APD), allopregnenolone (al-Preg), tetrahydrocortisol (TH-COL), tetrahydrocortisone (TH-COR), 11-β-hydroxyandrosterone (11-

OH-An), tetrahydrocorticosterone (THB), 3β,5α-tetrahydrodeoxycorticosterone(TH-DOC), tetrahydrodeoxycortisol (THS), 16-hydroxyestrone (16-OH-E1), 16-hydroxyestrone-2,3,4-$^{13}C_3$ (16-OH-E1-$^{13}C_3$), estriol-d_3 (E3-d_3), and tetrahydrocortisone-2,2,4,4,21,21-d_6-d_6 (TH-COR-d_6) were supplied by Toronto Research Chemicals (North York, Canada). Adrenosterone (AT), androsterone (An), androstenedione (AE), estrone (E1), and estradiol (E2) were purchased from Tokyo Chemical Industry (Tokyo, Japan). 7α-Hydroxydehydroepiandrosterone (7-OH-DHEA) and 7α-hydroxypregnenolone (7-OH-P5) 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 an 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.

2.2 | Human urine and ethical approval

The samples of healthy 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.

2.3 | Sample preparation

Steroid extraction strategy should be developed, taking into consideration the nature of the matrix, cleaning and pre-concentration step, and detection of conjugated steroid.[1,15,16]

Isolation of free steroids: 240 µL of urine was first mixed with the internal standard mixture solution (2.4 µL) (see Experimental section in the supporting information) and directly 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 × *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 (Figure S2).

Preparation of total steroids: 240 µL of urine was mixed with 240 µL of freshly prepared 0.15 M sodium acetate buffer (pH 4.6) containing 1.5 mg of L-ascorbic acid and 4 µL of β-glucuronidase-arylsulfatase solution. The solution was allowed to stand at 37°C for 16 h, which has been optimized in this study (see Figures S4 and S5), and then 960 µL of ACN was added 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 × *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% (v/v) ACN. The mixture was centrifuged at 19 000 × *g* for 15 min at 4°C and the supernatant was loaded on a Bond Elute C18 column (50 mg), treated similarly to that described above, and subjected to LC/MS/MS (Figure S3).

2.4 | LC/ESI-MS/MS

Ultrahigh-performance LC (UHPLC)/MS/MS was performed according to a 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 (2.1 × 100 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 40–80.0% B from 0 to 8min, maintained at 80% B from 8 to 10min, 80–40.0% from 10.0 to 10.10min, and held at 40% B from 10.1 to 13.1min. The injection volume was 20 µL. The SRM 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 0.2mM 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 a T-connector and passed through two in-line filters (Agilent 1290 inline filter, 0.3 µm), which were connected in tandem, prior to reaching the ion source (Figure S1). The auxiliary pump was off when conducting the "H method."

2.5 | Data processing

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

3 | RESULTS AND DISCUSSION

Free and total steroids in human urine were comprehensively analyzed using commercially available male and female urine. As discussed in a previous paper, 3-OH steroids undergo severe dehydration when ionized in positive ion mode.[14] Furthermore, additional OH group(s) in the backbone, such as 7-OH-DHEA and 7-OH-P5, further promote dehydration during ionization, making them hardly detectable by conventional LC/ESI-MS/MS (H method). Thus, the adduction of Li ion to steroid molecule in the ionization (Li method) could protect it from dehydration, resulting in an increase

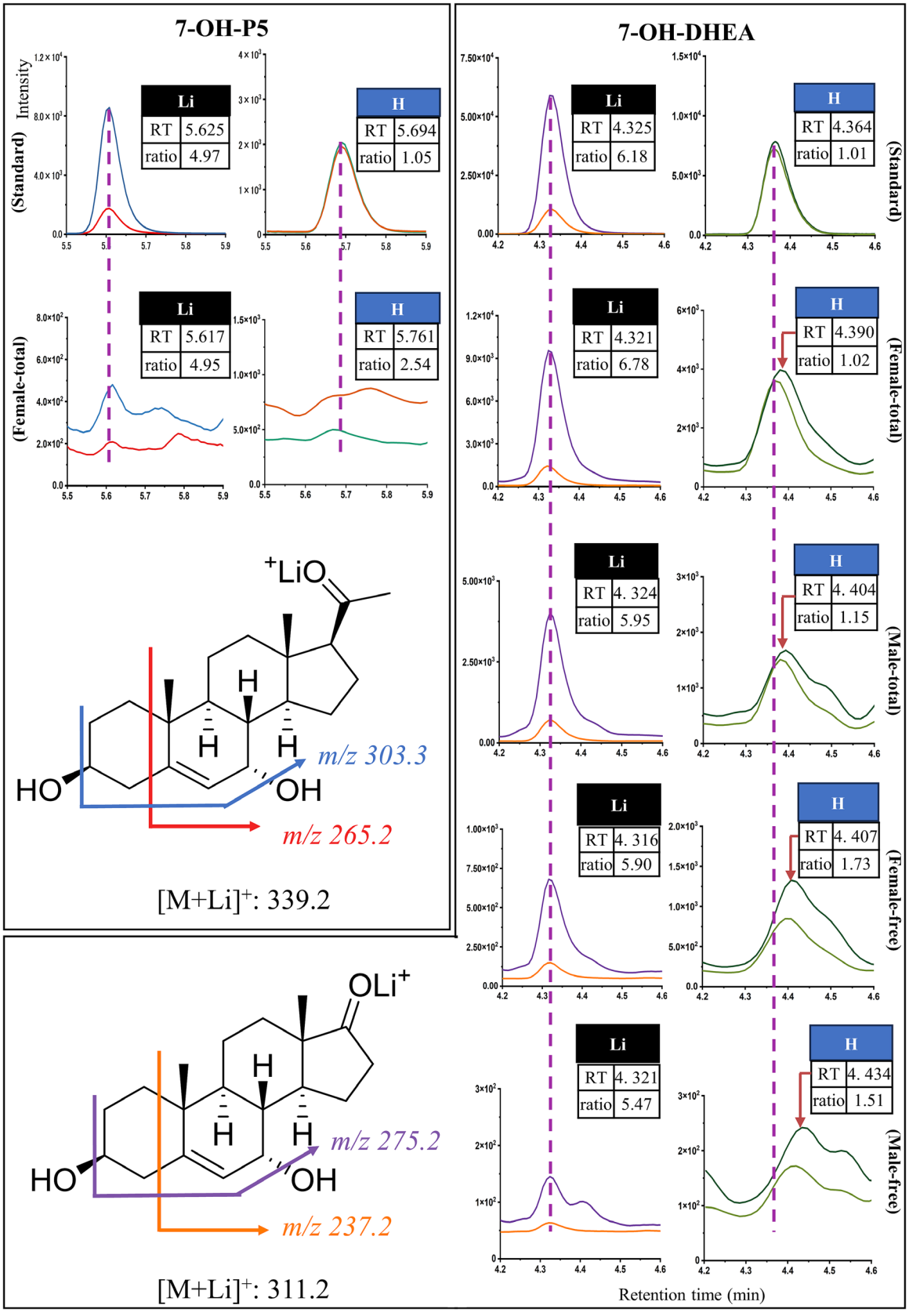

FIGURE 2 Legend on next page.

FIGURE 2 SRM 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), and male–free (fifth) by the Li method (left) and the H method (right). The values in each box indicate the retention time (upper) and peak-height ratio (lower) of the two peaks observed for the two SRM transitions listed in Table S1. The fragment ions used for the SRM transitions of 7-OH-P5 and 7-OH-DHEA are depicted in their structures [Color figure can be viewed at wileyonlinelibrary.com]

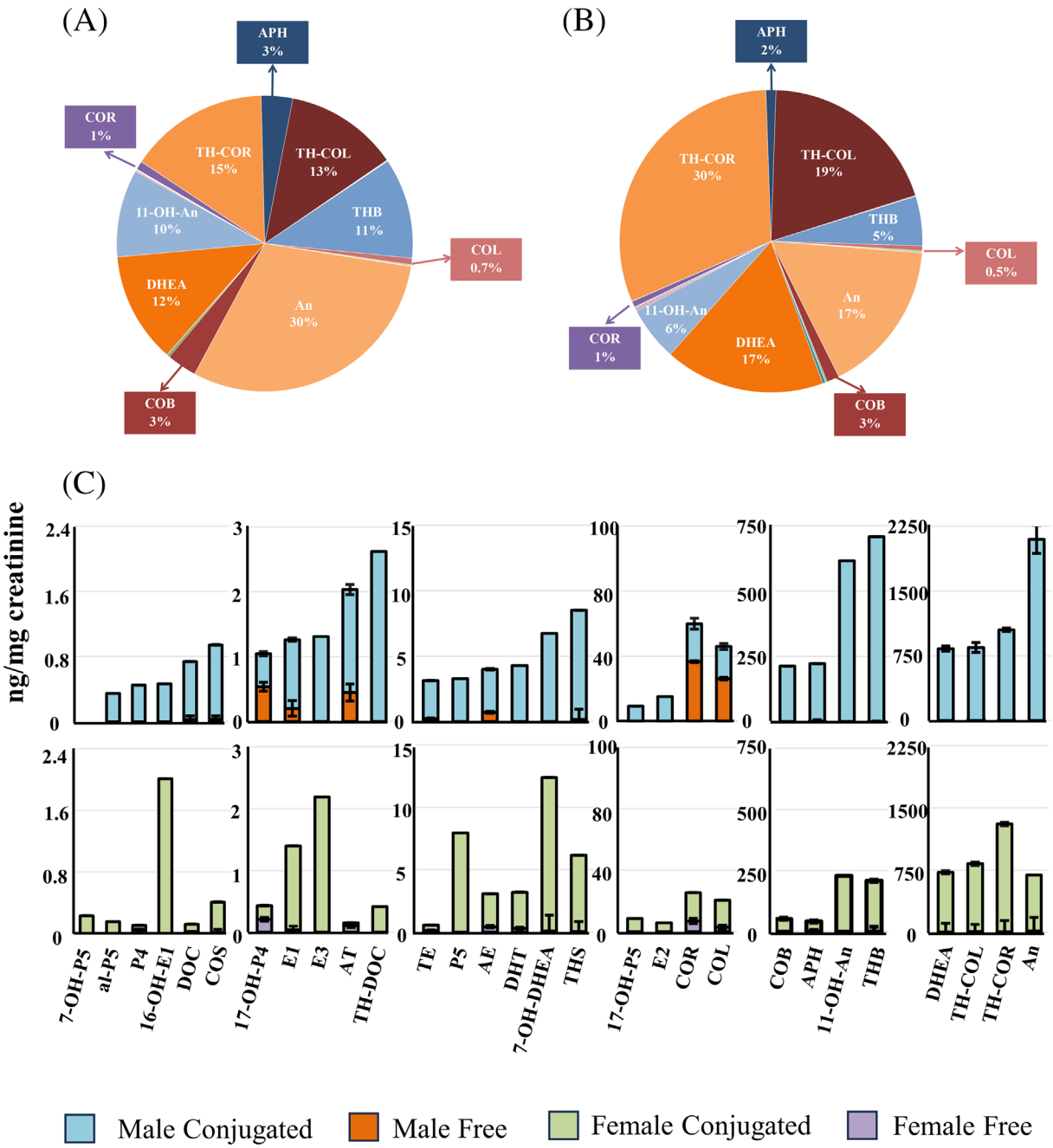

FIGURE 3 Pie charts of relative amounts of major steroids in male (A) and female (B) urine and bar chart of free and total steroid levels (ng/creatinine (mg)) obtained in male (top) and female (bottom) urine (C). All the data are summarized in Table S5 [Color figure can be viewed at wileyonlinelibrary.com]

in ion intensity of the precursor ion as a lithiated form and efficient generation of specific fragment ions for 3-OH steroids[14] (Figure 2; Figures S6 and S7, Table S1). On the other hand, other 3-ketosteroids without such easily dehydratable hydroxyl groups can be detected more efficiently by conventional LC/ESI-MS/MS than by the Li method, since it dilutes the column effluent twice with Li^+ solution prior to ionization. Since different types of steroids cause the above analytical problems, both the H and Li methods were used in this study in order to analyze as many types of steroids as possible. The LC/MS/MS system constructed in this study can easily switch between analytical settings for the H and Li methods, and can be operated continuously (Figure S1, supporting information).

Figure 2 compares SRM chromatograms of 7-OH-P5 and 7-OH-DHEA isolated from female and male specimens; 7-OH-P5 was only observed in total steroids of female specimens, while 7-OH-DHEA was observed in free and total steroids prepared from female and male samples. When these steroids were measured by the H method, 7-OH-P5 was barely observed and 7-OH-DHEA cannot be definitely assigned to the authentic peak due to an adjacent peak observed at a slightly slower retention time than the standard (Figure 2, right-hand panel). This could be ascribable to contamination of other unknown compounds. The adduction of a Li ion to steroid in the gas phase should protect it from dehydration or degradation during ionization. In fact, the sensitization ratios of the twelve 3-OH steroids, excluding 7-OH-DHEA, which could not be identified in the H method as described above, ranged from 1.2 to 7.4 (Figure S7).

Nineteen free and 29 total steroids were identified in human urine. The amounts of each steroid were normalized by creatinine values (Table S5). The majority of steroids were conjugated forms, and the composition of each steroid differed from that of free steroids, which accounted for about 1.2% of total steroids (Figure 3C). The predominant urinary steroids (conjugated forms) were TH-COL, TH-COR, APH, COB, THB, An, DHEA, and 11-OH-An. Female urine had relatively higher contents of 7-OH-P5, 16-OH-E1, E3, P5, and 7-OH-DHEA than male, while male urine had higher contents of DOC, AT, TH-DOC, TE, COR, COL, THB, 11-OH-An, and An, a result that clearly reflects gender differences (Figures 3A and 3B). Since 7-OH-P5, 16-OH-E1, E3, P5, 7-OH-DHEA, AT, TE, 11-OH-An, and An are sex steroids, it is normal for there to be significant differences between male and female urine samples. The stress-related corticosteroids DOC, TH-DOC, COR, COL, and THB were common in male urine[17]; however, this result needs to be confirmed with more specimens. The amount of each steroid was obtained only for a mixture of three samples each of male and female urine, and the levels were in approximately the same range as previously reported[2,18–25] (Table S5), which were obtained for individuals' urine or 24 h pooled urine.[22] Note that the present results did not reveal whether the stereoisomers corresponding to each steroid, such as An, etiocholanolone (Etio), and epiandrosterone (epiA); TE and epitestosterone (epiT); P5 and epipregnanolone (Epi-Preg); 7α-OH-DHEA and 7β-OH-DHEA, are present in the urine due to the limited number of authentic steroids on hand. However, it has been reported that the epimers in position 3 can be separated by LC/MS/MS[18,26] and that steroids containing stereoisomers can be analyzed more comprehensively by GC/MS,[27,28] although quantities are required due to the need for chemical derivatization.

4 | CONCLUSIONS

Lithium ion adduction-based UHPLC/MS/MS (Li method) was applied to urinary steroid analysis, enabling thirteen 3-OH steroids to be detected with higher sensitivity than using conventional LC/MS/MS (H method), of which 7α-OH-P5 was identified for the first time in human urine. In addition, combined with the H method, which detects protonated ions, 29 total steroids and 19 free steroids were identified in a limited volume (240 mL) of urine (Figure 1; Table S5). The profile of urinary steroids, which are predominantly conjugated forms, would be useful for monitoring relatively long-term physiological status or diagnosing chronic diseases.

AUTHOR CONTRIBUTIONS

Yue Pan: Investigation; conceptualization; formal analysis; writing—original draft; visualization. **Qiuyi Wang:** Investigation; conceptualization; formal analysis. **Mengyao Chen:** Investigation. **Toshifumi Takao:** Conceptualization; resources; funding acquisition; supervision.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1002/rcm.9719.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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