Rapid Identification of 44 Steroids in Human Urine Samples using HPLC-ESI-QTOF-MS

Running Title: Identification of 44 Steroids in Human Urine

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Abstract

Objective: Detailed analysis of un-processed and un-derivatized free and conjugated urinary steroids is useful to avoid miscalculations and to diagnose sports doping and adrenal problems including abnormal steroidogenesis, congenital deficiency of related enzymes, cancer, and other disease conditions. Hence, the present study was conducted to develop a soft ionization method to identify the maximum number of urinary steroids using ultra-performance liquid chromatography coupled with quadrupole time of flight mass spectrometer (HPLC–Q-TOF-MS).

Material and Methods: HPLC–Q-TOF-MS was carried out for the qualitative detection of steroids and their conjugates in urine samples. The method provides high sensitivity and fast analysis of steroids and their glucuronides without hydrolysis or sample preparation or extraction of steroids.

Results: Using the method, 44 steroids belonging to C-18, C-19, and C-21 classes and their conjugates were resolved and identified using positive and negative modes of ionizations by their characteristic ionization and collision energy induced dissociation behaviors.

Conclusion: The method is time-saving and good to compare samples from different peoples with control or healthy ones as it doesn't require any kind of pre-treatment or sample processing. It provides a complete picture of steroids metabolism and catabolism. It can be good for doping control or to explore the effects of other drugs. However, in qualitative analysis one may miss the significant information unless direct methods of steroids analysis to be employed.

Keywords: Steroids, Urine, HPLC, Q-TOF-MS, Qualitative Analysis

Introduction

Steroid hormones are synthesized in endocrine glands from cholesterol, hence are lipophilic molecules. Endocrine glands release the hormones into blood circulation where these acts as chemical messengers and act on a wide range of tissues to control the biological processes. Any disturbance in the synthesis of steroid hormones may result in various kinds of pathological conditions cancer, abnormal fertility, age-related diseases, and endocrine alterations. [1–4] Steroid hormones can be classified into four categories i.e. progestins, androgens, estrogens, and corticoids. The balance between free steroids and their conjugates are crucial to regulating their biological activity. Hence, the normal levels of these hormones are being sustained by their glucuronidation or sulfonation for easy excretion through urine.

A number of disorders and disease conditions are associated with steroid hormones. Hence, the analysis of steroid hormones is crucial in the initial stage of disease conditions. The most common methods to access the levels of steroids are radioimmunoassay (RIA), gas chromatography-mass spectrometry (GC–MS), and liquid chromatography-mass spectrometry (LC-MS). [5,6] Recent studies established the cross-reactivity of antibodies, hence RIA lacks specificity. [6] Y-survey conducted by College of American Pathologists Proficiency Testing Program in the year 2002 again confirmed that the antibodies in the commercially available kits lack specificity toward steroids. GC-MS is a very useful tool to analyze steroids in complex samples, however, steroids have to be derivatized and glucuronides and sulfated needs to be hydrolyzed before GC–MS analysis. Therefore, derivatization or hydrolysis may cause a problem and complicate sample treatment conditions. Moreover, after hydrolysis, it won't provide any information about the identification of steroid conjugates. [7,8]

LC-MS is easy and fast with high sensitivity and resolution. In LC-MS free as well as conjugated steroids can easily be analyzed. There are some published papers deal with the combined determination of glucuronides and free steroids. [9-11] APPI source has been reported to have good sensitivity and precision with CV <13%. [12,13] The methods were used to analyze patient samples and reported better than RIA. Tandem mass spectrometry has better specificity and is reported to have a correlation coefficient >0.0886. [14–16] It was reported to be correlated 6-β-hydroxycortisol, cortisol, hence human CYP3A activity, an important enzyme for drug metabolism. Current methods are based on HPLC or capillary LC-MS-MS having fewer resolution powers. Hence, currently available HPLC-Q-TOF-MS having a high resolution of more than 30000 and high accuracy may be more useful to analyze a greater number of steroids. Hence, in the current study, we utilized HPLC-Q-TOF-MS/MS for high selectivity and resolution at HPLC and Q-TOF-MS/MS levels for the qualitative analysis which further can be used for qualitative as well as quantitative analysis.

Materials and methods

Chemicals: Standards of steroidal compounds, their conjugates, and solvents i.e. water, acetonitrile, formic acid, were purchased from Sigma (St. Louis, MO). Internal and external calibrates and standards were purchased from Agilent Technologies. All other reagents used in the study were LC-MS grade.

Ethics statement

Human Ethics Committee of the PDDYP Ayurveda College, Pune, India approved the study wide letter no. RRI/2011/HEC/2023 dated 18-02-2011. While doing actual experimentation no deviations were made from the approved study protocol. This clinical trial was registered with the Clinical Trials Registry – India (CTRI) wide registration no. CTRI- 2016-08-007187.

Study subjects: Healthy males (n=10) volunteers were selected for the present study from the local areas of Pune, Maharashtra, India. The NRIBAS, Pune was the place for sample collection. All the volunteers have signed informed written consent. A physician generated the random allocation sequence, monitored the enrolment of participants. Volunteers having mass index >30 kg/m^2, blood pressure >160/90 mm Hg, present or prior history of cardiovascular diseases, diabetes mellitus, respiratory, gastrointestinal, hepatic, renal, endocrine, or reproductive disorders; or use of anti-hypertensive agents were excluded from the study.

Study design: Only male volunteers were selected. Subjects were kept under routine observation and checkups for one week before the actual trial phase (21- 28 March 2011). Subjects were provided breakfast, lunch, and dinner with two cups of tea in the daytime in the daycare center. After one week, urine samples were collected in tubes that had sodium azide (2.5 mM), centrifuged, filtered (0.2 µm filters), and were stored at -80°C till further analysis. Plasma fractions were separated from collected blood samples and were analyzed on a blood biochemical auto-analyzer (Sinnowa, China) (**Figure 1**).

UPC-QTOF-MS parameters: Urine samples were diluted with acetonitrile: water (80:20 v/v) in a 1:4 ratio, centrifuged at 8000 rpm for 5 min at 15ºC (9). Samples (20µl) were injected into the HPLC (Agilent 1290 Infinity Series) fitted with ZORBAX 300SB C-18 (4.6 x 150 mm, 5.0µm particle size) and interfaced to Agilent 6538 Accurate-Mass Q-TOF-MS to resolve urine samples. The mobile phase had (A) 0.1% formic acid in water and (B) 0.1% formic in acetonitrile: water (80:20). Mobile phase A 5% was in the isocratic mode for the first 5 minutes and then increased to 95 % in a linear gradient mode up to 40 min. It decreased up to 5% at 45 min and was remained constant till 50 min. The flow rate of the mobile phase was 0.3 ml/min. Parameters of Q-TOFMS were optimized for the positive and negative ion polarity mode. It was extended to dynamic mode (1700*m/z*, 2GHz) with fragmentor voltage 195V, ramped collision energy from 10 to 40 eV, and 3 spectra s^{-1} acquisition rate. Continuous internal calibration was maintained during the data collection.

Identification of steroids: Steroids present in the samples were identified using standards as well using standard libraries and matching the characteristic ion peaks reported in literature and present in the extracted MS/MS spectra. A CSV file was made from identified steroids containing exact monoisotopic mass and retention time to extract the recursive features from the mass data obtained from the samples collected from healthy groups.

Statistical Analysis: The data were analyzed using one-way analysis of variance (ANOVA) combined with Tukey's multiple comparison test, or the Student's *t*-test, when appropriate. Differences between groups were considered significant at $P < 0.05$ and values were reported as means ± SE.

Results

Clinical Biochemistry: Values of clinical biochemistry parameters analyzed in this study were expressed as mean ± standard deviation **(Table 1)**.

Resolution and identification of urinary steroids: Present method resolved the steroids from 21 to 36 minutes in unprocessed urinary samples. It was observed that the addition of 0.1% formic acid efficiently ionized the compounds as compared to other formate buffers. Some of the urinary steroids showed a resolution difference of milliseconds. However, further resolution in TOF helped to resolve and identify the urinary steroids. Steroids are also poorly soluble in polar solvents and therefore in urine, very minute quantities are present. Hence, a greater sensitivity of MS/MS is required. In the present study, 44 steroids were tentatively identified with their ion abundance. Standards of cortisol corticosterone testosterone estrone, estradiol, etc. (250 ng/mL) were used in the mixture as well to spike the samples. A sum of free and conjugated steroids was considered for the isomeric steroids i.e. testosterone/epitestosterone, androsterone/etiocholanolone and 5α-androstane-3α, 17β-diol/β- androstane-3α, 17β-diol despite their individual identifications. Initially, all the steroids were identified using their monoisotopic masses. Compounds having the same isotopic masses (± 20 ppm) as listed in KEGG for the compounds present in the steroids hormones biosynthesis pathway were listed out and subjected to further confirmation by comparing with standard spectra of these compounds. Finally, 44 compounds were found to have the best match with standard spectra (**Table 2**). Four compounds i.e. 2-methoxyestrone 3-glucuronide, 11-ketoetiocholanolone, 19-hydroxytestosterone, and 16α-hydroxy-dehydroepiandrosterone elute from 26.5 To 26.7 min formed the largest peak due to the merged peak of all four compounds, however, in real-time, these were well separated and further resolved in TOF. Three compounds namely deoxycortisol, tetrahydrocorticosterone, and dihydrocortisol also eluted in a narrow period of 27.7 to 27.8 min but being compounds of different molecular weight and resolution by TOF these could be separated and identified. Cortisone and aldosterone were also found to co-elute but again due to different masses, these could be separated in TOF and identified. Another three compounds i.e. pregnenolone, pregnanediol, and 5β-dihydrotestosterone also co-elute at 33.7 min but well-resolved at TOF level and identified. All other or 30 steroids were well resolved in the HPLC conditions used in the study (**Figure 2**).

Confirmation of identification using MS/MS: The identification of 44 steroids enlisted in table 1 were further confirmed using standard spectra of these compounds and confirmation of characteristic ion peaks reported in the literature. **Table 3** is showing the ion peaks observed for different compounds along with characteristic ion peaks in bold. All the MS/MS spectra of these 44 steroids are shown in **supplementary file 1**. ESI Spectra of these steroids characterized by stable MH^+ and product ions MH^+-H_2O, MH^+-$2H_2O$, and extensive water loss. The relative abundance of MH+ was observed to be high in the case of the ion at m/z 317.240 with insignificant isomeric compounds. Its structure was tentatively assigned to pregnenolone by comparing the monoisotopic mass and their characteristic products ions at *m/z* 299 $[M\text{-}H_2O]^+$, 281 $[M\text{-}2H_2O]^{+\cdot}$ and 159. Comparison of MH+ ion at m/z 347.214 and its product ions at *m/z* 329 $[M\text{-}H_2O]^+$, 311 $[M\text{-}2H_2O]^{+\cdot}$ and 121 with standard spectra establish its structure to corticosterone. Product ions at *m/z* 311 and 121 were reported to specific ions generated through the breakdown of corticosterone. MH+ ions at *m/z* 363.209 gave product ions at *m/z* 345 $[M\text{-}H_2O]^+$, 327 $[M\text{-}2H_2O]^+$, 309 $[M\text{-}2H_2O]^+$ and 121. These product ions were similar to corticosterone with one additional hydroxyl group, hence structure was established as 6-hydroxycorticosterone. Protonated ion at *m/z* 331.219 [M+H]+ form daughter ions at *m/z* 313 $[M\text{-}H_2O]^+$, 295 $[M\text{-}2H_2O]^+$, 271, 253, 121, and 109 was identified as deoxycorticosterone. Protonated parent ion of *m/z* 351.247 generated specific ions at *m/z* 333 $[M\text{-}H_2O]^+$ and 169 was tentatively assigned as tetrahydrocorticosterone based on isomeric mass and fragments. Protonated ion $[M+H]^+$ at *m/z* 321.271 fragments into *m/z* 303 $[M\text{-}H_2O]^+$, 285$[M\text{-}H_2O]^+$, 275 $[M\text{-}H_2O\text{ -}CO]^+$, 189, 175,153, 121 and 107. When compared with standard mass libraries, its identification was established as pregnanediol, further confirmed with related literature. MH+ of *m/z* 347.214 breakdown further into *m/z* 329 $[M\text{-}H_2O]^+$, 311 $[M\text{-}2H_2O]^+$, 299 $[M\text{-}H_2O\text{ -}CO]^+$, 253, 189, 175, 149 and 97, the characteristic product ions of deoxycortisol. Parent ion $[M+H]^+$ at *m/z* 363.209 showed the sequential water losses at *m/z* 345, 327, and 309. More products at *m/z*

345, 327, 309, 269, 199, 175, 121 and 109 confirmed its identity as cortisol. Another protonated ion at 365.224 produced product ions in a similar fashion as cortisol with increased mass by 2 Da i.e. *m/z* 347; 329, 311 and same as cortisol i.e. at *m/z* 269, 175, 121 and 107, hence the identity of compounds was established as dihydrocortisol, Similarly, tetrahydrocortisol was identified as it showed parent ion at *m/z* 367.240 and product ions at *m/z* 349, 331, 313 increased by 4Da to that of cortisol and 257, 199, 187, 121, 109 similar to cortisol. MH^+ ion at *m/z* 361.193 gave product ions at *m*//z 343 $[M\text{-}H_2O]^+$, 325 $[M\text{-}2H_2O]^+$ 307 $[M\text{-}3H_2O]^+$ whereas product ions at *m/z* 301 and 163 are characteristic product ions of cortisone. Similarly, $[M+H]^+$ ion of 365.227 Da produced product ions at *m/z* 347 $[M\text{-}H_2O]^+$, 329 $[M\text{-}2H_2O]^+$, 311 $[M\text{-}3H_2O]^+$ as produced by cortisone but increased by 4 Da and product ions at *m/z* 243, 175 and 121, hence its identification was established to tetrahydrocortisone. Protonated ion of *m/z* 367.196 similarly gave product ion to tetrahydrocortisol but missing product ion at 163 and prominent peaks at 133, 121, and 109 lead its identification as cortolone (**Figure 3**).

Protonated $[M+H]^+$ ion of 303.201 Da and its fragment ions at *m/z* 285 $[-H_2O]$, 267 $[-H_2O]$, 257, 189, 161, 135, 121, 109 were attentively assigned as 16α-hydroxyandrost-4-ene-3, 17-dione. The protonated precursor ion of 287.201 Da exhibited two subsequent neutral water losses and formed peaks at *m/z* 269, 251 along with fragments at *m/z* 203 and 185 characteristic fragments of androstenedione. The precursor ion of $[M+H]^+$ 291.224 da resolved at 32.9 min fragmented into mass ion at *m/z* 255, 199, and 147, characteristic ions of etiocholanolone. Another protonated compound of 467.275 Da produced product ion at *m/z* 291 due to loss of glucuronide with other product ions at *m/z* 273 $[-H_2O]$, 255 $[-H_2O]$, 123, 111 was tentatively assigned as etiocholan-3α-ol-17-one 3-glucuronide. Structure of androsterone [M+H]+ of isotropic mass 291.224 Da was confirmed due to mass ions at *m/z* 273 $[-H_2O]$, 255 $[-H_2O]$, 199, and 147. Androsterone glucuronide (467.259 Da) was identified due to characteristic mass ions at *m/z* 449, 291 [-glucuronide], 273 $[-H_2O]$, and 255. The protonated $[M+H]^+$ precursor ions of 307.219 and 483.264 gave products ions at *m/z* 271, 261, 253, 199, 149, 145, 135, 121, and at *m/z* 307, 289 were assigned to 11-hydroxyandrosterone and 11-β-hydroxyandrosterone-3-glucuronide respectively. Protonation of 16α-hydroxy-dehydroepiandrosterone formed product ion peaks at *m/z* 287 $[-H_2O]$, 269 $[-H_2O]$, 245, 243, 135, 125, and 109. Testosterone was tentatively identified by its monoisotopic mass of 289.208 $[M+H]^+$ and product ion peaks at *m/z* 271, 253, 189, 147, 109, 97. It was in accordance with Kobayashi et al as testosterone mass spectrum contained major peaks as MH^+ and 7% $MH^+\text{-}H_2O$. Dehydroepiandrosterone (DHEA) gave the same peaks as testosterone, however by matching with standard, it was found to have a low-intensity product peak at *m/z* 271 and prominent peaks at *m/z* 171 and 161 as compared to testosterone. 5β-Dihydrotestosterone (precursor ion at *m/z* 291.259) was identified by comparing its product ion peaks at *m/z* 273 $[-H_2O]$, 255 $[-H_2O]$, 247, 217, 159, 111, 109, and 105. Further confirmation of 5β-dihydrotestosterone structure was due to a relatively high abundance of MH^+ insignificant isomeric compounds. Structure 19-hydroxytestosterone was assigned due to formation of product ion peaks at *m/z* 287 $[-H_2O]$, 269 $[-H_2O]$, 109; androstane-3,17-diol at *m/z* 275, 265, 257, 253, 247, 155, 125, 109, 105; and testosterone glucuronide at *m/z* 447 $[-H_2O]$, 411, 289 [-glucuronide], 271 $[-H_2O]$, 253 $[-H_2O]$, 243, 189, 175, and 109 (**Figure 3**).

Estrone and estrone-3-sulfate were identified in negative mode by assigning the product mass ion peaks at *m/z* 251, 207, 145, 127, and at *m/z* 271, 269 respectively. Intense MH^+ peak due to the presence of a hydroxy group in the aromatic ring also confirmed the compound as estrone. Estrone glucuronide was identified in positive ion mode from the product mass ion peaks at *m/z* 429, 411, 271 (due to loss of glucuronide; -176 Da), 253, 227, 130, 125, and 101. Product ion spectra of [$M+H^+$; *m/z* = 287.156] showed that *m/z* 269, 251 were originated from water loss, whereas *m/z* 161, 147, 133, 123, 109 are the characteristic product ions of 2-hydroxyestrone. Product ions of [M+H]+; 301.178] at *m/z* 283 $[M\text{-}H_2O]^+$, 273, 187, 165, 149, 125 help to tentatively assign the structure to 2-methoxyestrone. Another precursor ion at *m/z* 477.209 formed major product ions at *m/z* 301 (loss of glucuronide i.e. 176 Da), and 283 lead to assign the structure as 2-methoxyestrone 3-glucuronide. The parent ion of *m/z* 378.129 resolved at 27.8 min in negative ionization mode break into product ions of *m/z* 299, 271, and 199 that established its structure as 2-methoxyestrone 3-sulfate. Parent ion resolve at 33.3 min of *m/z* 273 generated product ions of *m/z* 255,

183, 161, 159, 133, and 107 was equivalent to estradiol, when compared to its standard mass spectra. The insignificant $[M+H]^+$ ion and most abundant $[M+H -2H_2O]^+$ ion further confirmed the structure of estradiol. 17 β-Estradiol 3- (β-D-glucuronide) was resolved at 25.2 min and gave product ions of *m/z* 273, 255, and 159. Moreover, the presence of hydroxyestradiol in urine samples was established due to the presence of parent ion at *m/z* 271 $[M-H_2O]^+$ and product ions at *m/z* 253 $[M-H_2O]^+$, 161, 135, and 107. Further, its glucuronide i.e. hydroxyestradiol glucuronide (*m/z* 465) was found to be resolved at 30.2 min and having product ions at *m/z* 271, 255, 175, 165, 131, 109, and 107 (**Figure 3**).

The protonated parent ion of *m/z* 465.248 generated product ions at *m/z* 289 [loss of glucuronide], 271 [M-Glu $-H_2O]^+$, 253 [M–Glu $-2H_2O]^+$, 189, 157, and 135 that tentatively established its structure as 16α, 17β-estriol 3- (β-D-glucuronide). Precursor ion of *m/z* 479.220 generated product ions at 303 [loss of glucuronide], 285 $[M-H_2O]^+$, 251, 217, 205, and 149 was tentatively assigned as 2-methoxyestradiol 17β-3-glucuronide. The structure of epiandrosterone (*m/z* 291.224) was established due to its characteristic product ions at *m/z* 273 $[M-H_2O]^+$, 255 $[M-2H_2O]^+$, 159, 135, 133, 107, and 105 as compared to its standard spectra. A precursor ion of *m/z* 467.149 was tentatively identified as 6-dehydrotestosterone 17-glucosiduronic acid due to product ions at *m/z* 287 [-180 Da, due loss of glucosiduronic acid], 269 $[M-H_2O]^+$, 189, 121, 111, 109, and 107. Glucuronide of dehydroepiandrosterone (*m/z* 463.262) was identified due to its product ions at *m/z* 293 [-glucuronide], 291, 273 $[-H_2O]$, 255 $[-H_2O]$, and 107. Precursor ion at *m/z* 361.195 was identified as aldosterone as it showed fragments at *m/z* 343 $[M-H_2O]^+$, 333 $[M-CO]^+$, 315 $[M -2H_2O -CO]^+$, 301, 163, 121, 107, and 105 (**Figure 3**). The various identified urinary metabolite was further explored via KEGG pathway to predict the effect of changes in metabolite levels on the human physiology (**Figure 4)**.

Discussion

Soft ionization methods (ESI) are efficient to detect steroidal hormones despite their structural similarities. In the current study, ESI-QTOF-MS enabled to identify 44 steroids in a single of urine sample of healthy peoples. Adrenal hormones synthesis starts from cholesterol to pregnenolone. Pregnenolone is the precursor of all the C_{18}, C_{19}, and C_{21} steroids. Most of the methods, being used for sample processing or derivatization usually lead to hydrolysis of conjugated steroids and hence mislead the calculations. [17–19] The major issues to analyze steroids with LC-MS/MS are matrix effects, ionization efficiency, and isobaric interferences. [20,21] Steroids like 17-hydroxypregnenolone, pregnenolone, and DHEA reported being less ionized with soft ionization techniques or normal LC-MS/MS techniques. Various derivatization techniques are being employed to ionize and resolve the steroids. The resolution and ionization of 44 steroids in a single run proved that soft ionization source (ESI) is the most suitable to produce specific diagnostic product ions. Moreover, the derivatization needs extra time and labor and may lead to hydrolysis of conjugates, hence precision may be compromised.

One important factor that needs to be mentioned that all 44 steroids were not present universally among all the samples. This finding indicates the different synthesis or catabolism rate of steroidal hormones in healthy individuals. [22–24] Hence, from qualitative data, where ion abundances were noticed can be helpful to identify the abnormal catabolism or synthesis of steroids. In previous studies, free forms of steroids were ignored and more emphasis was given to conjugated steroids ratio. However, free forms of steroids excreted in the urine are more relevant to diagnose the enzyme deficiencies. [25] Moreover, relative abundances of free steroids to their conjugates at the same time without hydrolysis and conjugation before the chromatographic separation may help in accurate determination of the ratios of the conjugates and reduce the risk of false positives and misleading results. The relative ratio of steroidal hormones may indicate the predisposition of individuals to various kinds of disorders or diseases such as inflammation or neurodegenerative disorders and multiple sclerosis as studies showed that testosterone and estrogens are neuroprotective and anti-inflammatory agents. [26–28]

CONCLUSION

Overall, the study emphasizes the use of soft ionization for qualitative and quantitative analysis of steroidal hormones. In the current study, the protocol developed to resolve and to identify steroids qualitatively using soft ionization of steroids. Sample preparation doesn't require any treatments, hence it is fast. The method can further be used to quantify these steroids or to compare their levels in urine samples qualitatively. Relative abundances or quantity may be used to diagnose people with abnormal steroidogenesis, congenital deficiency of related enzymes, cancer, neurodegenerative, oxidative stress, and other disease conditions.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Human Ethics Committee of the PDDYP Ayurveda College, Pune, India approved the study wide letter no. RRI/2011/HEC/2023 dated 18-02-2011. While doing actual experimentation no deviations were made from the approved study protocol. This clinical trial was registered with the Clinical Trials Registry – India (CTRI) wide registration no. CTRI- 2016-08-007187.

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. All the experiments on animals were done according to the guidelines.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Table 1: Lipid, glucose and uric acid profiles of health males (mean ± SD).

S. No.	Parameters	HLT
1.	Age (years)	39.2±2.4
2.	BMI	22.1±-2.2
3.	TG (mg/dl)	98.36±3.01
4.	TC (mg/dl)	155.13±4.43
5.	HDL (mg/dl)	45.10±2.52
6.	LDL (mg/dl)	108.50±6.11
7.	VLDL (mg/dl)	18.26±3.65
8.	Uric acid (mg/dl)	4.93±0.91
9.	Glucose (mg/dl)	97.06±7.06

Note * Mark expressed the significant differences i.e. $p<0.05$.

Table 2: Table is showing mass, retention time, and differences in the monoisotopic mass observed (in ppm) along with the ion abundances of urinary steroids. Ion abundance for the compounds detected in negative ion polarity mode is not shown in the table.

S. No.	Tentative Compound	KEGG	Mass	RT	Polarity	Formula	Diff (ppm)	Abundance
1.	Estrone	C00468	268.152	24.9	-ve	$C_{18}H_{22}O_2$	3.6	-
2.	Estradiol	C00951	272.177	33.3	+ve	$C_{18}H_{24}O_2$	2.3	27832
3.	2-Hydroxyestrone	C05298	286.156	29.2	+ve	$C_{18}H_{22}O_3$	3.1	11360
4.	Androstenedione	C00280	286.201	29.2	+ve	$C_{19}H_{26}O_2$	0.4	11925
5.	Testosterone	C00535	288.208	31.5	+ve	$C_{19}H_{28}O_2$	3.2	8038
6.	Dehydroepiandrosterone	C01227	288.208	31.6	+ve	$C_{19}H_{28}O_2$	3.2	11852
7.	Etiocholanolone	C04373	290.224	32.9	+ve	$C_{19}H_{30}O_2$	2.0	9654
8.	Androsterone	C00523	290.224	33.0	+ve	$C_{19}H_{30}O_2$	2.0	27889
9.	Epiandrosterone	C07635	290.224	33.6	+ve	$C_{19}H_{30}O_2$	2.0	9538
10.	5β-Dihydrotestosterone	C05293	290.259	33.7	+ve	$C_{19}H_{30}O_2$	2.0	7063
11.	Androstane-3,17-diol	C12525	292.240	30.8	+ve	$C_{19}H_{32}O_2$	0.9	21133
12.	2-Methoxyestrone	C05299	300.178	35.6	+ve	$C_{19}H_{24}O_3$	7.8	14850
13.	16α-Hydroxyandrost-4-ene-3,17-dione	C05140	302.201	36.2	+ve	$C_{19}H_{26}O_3$	16.4	23509
14.	11-Ketoetiocholanolone	C14552	304.203	26.6	+ve	$C_{19}H_{28}O_3$	2.8	11685
15.	16α-Hydroxy-dehydroepiandrosterone	C14606	304.203	26.7	+ve	$C_{19}H_{28}O_3$	2.8	12522
16.	19-Hydroxytestosterone	C05294	304.204	26.6	+ve	$C_{19}H_{28}O_3$	-0.5	10038
17.	11-Hydroxyandrosterone	C14606	306.219	32.8	+ve	$C_{19}H_{30}O_3$	1.6	22992
18.	Pregnenolone	C01953	316.240	33.7	+ve	$C_{21}H_{32}O_2$	0.7	21241
19.	Pregnanediol	C05484	320.271	33.7	+ve	$C_{21}H_{36}O_2$	1.6	7074
20.	Deoxycorticosterone	C03205	330.219	27.4	+ve	$C_{21}H_{30}O_3$	1.5	11799
21.	Corticosterone	C02140	346.214	26.9	+ve	$C_{21}H_{30}O_4$	1.2	39664
22.	Deoxycortisol	C05497	346.214	27.7	+ve	$C_{21}H_{30}O_4$	1.2	76521
23.	Estrone 3-sulfate	C02538	348.204	31.1	-ve	$C_{18}H_{22}O_5S$	-	-
24.	Tetrahydrocorticosterone	C05476	348.257	27.8	-ve	$C_{21}H_{34}O_4$	2.0	-
25.	Cortisone	C00762	360.193	29.0	+ve	$C_{21}H_{28}O_5$	1.8	15041
26.	Aldosterone	C01780	360.199	29.0	+ve	$C_{21}H_{28}O_5$	6.9	6457
27.	Cortisol	C00735	362.209	28.8	+ve	$C_{21}H_{30}O_5$	0.9	7986
28.	6-hydroxycorticosterone	C01124	362.209	31.9	+ve	$C_{21}H_{30}O_5$	0.9	11071
29.	Dihydrocortisol	C05471	364.224	27.8	+ve	$C_{21}H_{32}O_5$	2.7	16416
30.	Tetrahydrocortisone	C05470	364.227	27.0	+ve	$C_{21}H_{32}O_5$	1.4	17225
31.	Cortolone	C05481	366.196	28.0	+ve	$C_{21}H_{34}O_5$	1.7	6419
32.	Tetrahydrocortisol	C05472	366.240	28.0	+ve	$C_{21}H_{34}O_5$	3.2	15041
33.	2-Methoxyestrone 3-sulfate	C08358	378.129	28.7	-ve	$C_{19}H_{24}O_6S$	19.7	-
34.	Estrone glucuronide	C11133	446.193	23.3	+ve	$C_{24}H_{30}O_8$	19.8	6152
35.	17 β-estradiol 3- (β-D-glucuronide)	C05503	448.209	25.2	+ve	$C_{24}H_{32}O_8$	1.6	10197
36.	6-Dehydrotestosterone 17-glucosiduronic acid	-	462.262	31.7	+ve	$C_{25}H_{34}O_8$	-	25547
37.	Testosterone glucuronide	C11134	464.226	30.5	+ve	$C_{25}H_{36}O8$	0.1	15487
38.	Hydroxyestradiol glucuronide	-	464.241	30.2	+ve	$C_{25}H_{36}O_8$	0.1	30252
39.	16α, 17β- Estriol 3- (β-D-glucuronide)	C05504	464.248	30.1	+ve	$C_{24}H_{32}O_9$	1.4	43240
40.	Androsterone glucuronide	C11135	466.259	22.3	+ve	$C_{25}H_{38}O_8$	11.7	11161
41.	Etiocholan-3α-ol-17-one 3-glucuronide	C11136	466.258	23.9	+ve	$C_{25}H_{38}O_8$	11.6	12079
42.	2-Methoxyestrone 3-glucuronide	C11132	476.209	26.5	+ve	$C_{25}H_{32}O_9$	7.2	15096
43.	2- Methoxyestradiol 17β-3- glucuronide	C11131	478.220	31.7	+ve	$C_{25}H_{34}O_9$	0.6	13637
44.	11-β-hydroxyandrosterone-3-glucuronide	-	482.264	21.5	+ve	$C_{25}H_{38}O_9$	9.5	2414

Table 3: Metabolites identified from their MS/MS product ions in the urine samples from healthy individuals. Compounds in the table are arranged in the fashion they appear in the synthesis cycle.

S. No.	Tentative Compound	m/z	RT	Characteristic mass ions
1.	Pregnenolone	317.240	33.7	**299**, **281**, 271, 257, 231, 217, 205, **159,** 137, 121, 109
2.	Corticosterone	347.214	26.9	**329**, 311, 299, 271, 253, 243, 225, 175, 161, 149, **121,** 109
3.	6-hydroxycorticosterone	363.209	31.9	**345, 327,** 309,297, 267, **269,** 253,199, 187, 175, 147, 135 **121,** 109
4.	Deoxycorticosterone	331.219	27.4	**313**, 169
5.	Tetrahydrocorticosterone	351.247	22.5	333, 307, **305**, 289, 287, **281**, **267**, 245, 237, 215, 193, 175, **113**
6.	Pregnanediol	321.271	33.7	**303**, 285, **275**, 259, **251,** 191, 179, 173, 163**, 158,** 137, 125, 107
7.	Deoxycortisol	347.214	27.7	**329**, **311**, 299, 271, 253, 223, 211, 189, **175**, 149, 135, **109**
8.	Cortisol	363.209	28.8	**345,** 327, 309, **297,** 291, 283, **269, 267**, 241, 199, 175, 147, 135, 121, **109**
9.	Dihydrocortisol	365.224	27.8	**347**, 329, 311, **299**, 283, 281, **269**, 257, 251, 241, 225, 187, 159, 135, **121**, **107**
10.	Tetrahydrocortisol	367.240	28.0	349, 331, 309, 289, 275, 257, 199, 187, 121, 109
11.	Cortisone	361.193	29.0	345, **301**, 283, 247, 219, 205, **163**, 147, **121,** 111, **107**
12.	Tetrahydrocortisone	365.227	27.0	**347, 329, 311**, 299, **269,** 243, 197, 191, 149, 121, 109
13.	Cortolone	367.196	28.0	**349**, 331, **309**, 289, 287, **275**, 257, 249, 213, 199, 187, 151, 135, **133**, **121**, 109
14.	16α-Hydroxyandrost-4-ene-3,17-dione	303.201	36.2	**285**, 267, 257, 247, 229, 207, **189**, **161**, **135, 121, 109**
15.	Androstenedione	287.201	29.2	**269**, 251, 245, **229**, 171, 159, **109**
16.	Etiocholanolone	291.224	32.9	273, **255**, 245, 227, **215**, **199**, 177, 159, 145, 121, **109**, 105
17.	Etiocholan-3α-ol-17-one 3-glucuronide	467.275	23.9	449, **291**, **273**, 123, 111
18.	Androsterone	291.224	33.0	273, **255**, 245, 227, 226, **215, 199**, 149, 147, **133, 121**, 109, **107**
19.	Androsterone glucuronide	467.259	22.3	449, **291, 273**, 255, 237, 229, 217, 201
20.	11-Hydroxyandrosterone	307.219	32.8	**289, 271**, 261, 253, 243, 235, **225**, 219, 199, 197, 187, **177**, 165, **149**, **145**, 143, 137, **135**, **121**, 119, 109, 107
21.	11-β-Hydroxyandrosterone-3-glucuronide	483.264	21.5	307, 289
22.	Estrone	269.152	24.9	251, 233, 207, **191, 145, 127**
23.	Estrone glucuronide	447.193	23.3	429, 411, 271, 253, 227, 130, 125, 101
24.	Estrone 3-sulfate	349.204	31.0	271, **269**
25.	2-Hydroxyestrone	287.156	29.2	**269, 251**, 229, 185, 157, 147, **133, 123, 109**
26.	2-Methoxyestrone	301.178	35.6	**283**, 273, 255, 215, 203, 189, 177, **165**, 149, 125
27.	2-Methoxyestrone 3-glucuronide	477.209	26.5	**301**, 283,
28.	2-Methoxyestrone 3-sulfate	379.129	28.7	**299**, 271, 199

29.	16α-Hydroxy-dehydroepiandrosterone	305.203	26.7	287, **269**, 245, **243**, 215, 191, 171, 159, **135**, 125, **109**
30.	Dehydroepiandrosterone	289.208	31.6	**271**, 253, 175, 171, 135, 121**, 109**
31.	Testosterone	289.208	31.5	271, **253**, 189, 147, **109**
32.	5β-Dihydrotestosterone	291.259	33.7	273, **255**, 247, 239, 233, **217**, 203, 189, 177, 151, **149**, 135, 125, **123**, 111, **109**, **105**
33.	19-Hydroxytestosterone	305.204	26.6	**287**, **269**, 243, 233, 231, 211,**189**, 175, 147, **133**, 125, **109**
34.	Androstane-3,17-diol	293.240	30.8	**275, 265, 257, 253, 247**, 229, 183, 179, 177, 169, **163**, 155, 145, **133, 125**, 117, 115, **109, 107**, 105
35.	Testosterone glucuronide	465.226	30.5	447, 411, **289, 271**, 253, 243, **189, 175**, 161, 141, 123, 109
36.	Estradiol	273.177	33.3	**255**, 227, **215, 199**, 173, 161, 159, **147**, 145, 135, **133**, 121, 107
37.	17 β-estradiol 3- (β-D-glucuronide)	449.209	25.2	**273, 255, 227, 203**, 175, 147, 125, 121
38.	Hydroxyestradiol glucuronide	465.241	30.2	**271, 255, 253**, 245, 213, 197, 189, 185, **175, 171**, **165**, **161**, 149, 145, 135, 131, 125, 123, 117, **109, 107**
39.	16α, 17β- Estriol 3- (β-D-glucuronide)	465.248	30.1	**289, 271**, 253, 189, **161, 135**
40.	2- Methoxyestradiol 17β-3-glucuronide	479.220	31.7	**303, 285, 251, 217, 205**, 149
41.	Epiandrosterone	291.224	33.6	273, **255**, 245, 215, **199**, 185, **173, 159**, 147, **135, 133**, **107, 105**
42.	6-Dehydrotestosterone 17-glucosiduronic acid	467. 149	22.3	**287, 269**, 251, 241, 229, 213, 201, **189**, 171, 159, 147, 135, 131, **121**, 111, 109, **107**
43.	Dehydroepiandrosterone 3-glucuronide	463.262	31.7	**293**, 291, 273, 231, 219, 141, 139, 109
44.	Aldosterone	361.195	29.0	**343, 333, 315,** 301, 283, 257, 187, 171, **163,** 139, **121, 107, 105**`

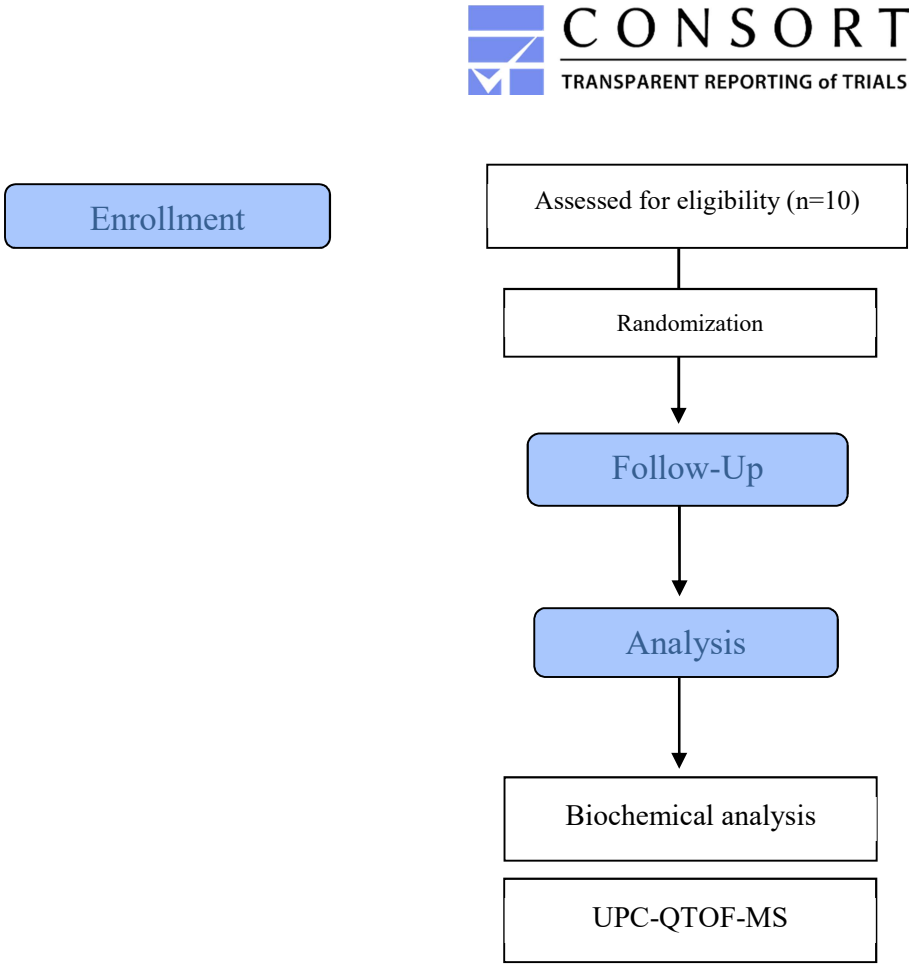

Figure 1: A CONSORT flow diagram of study design, sample collection, data processing, and data analysis in different study groups.

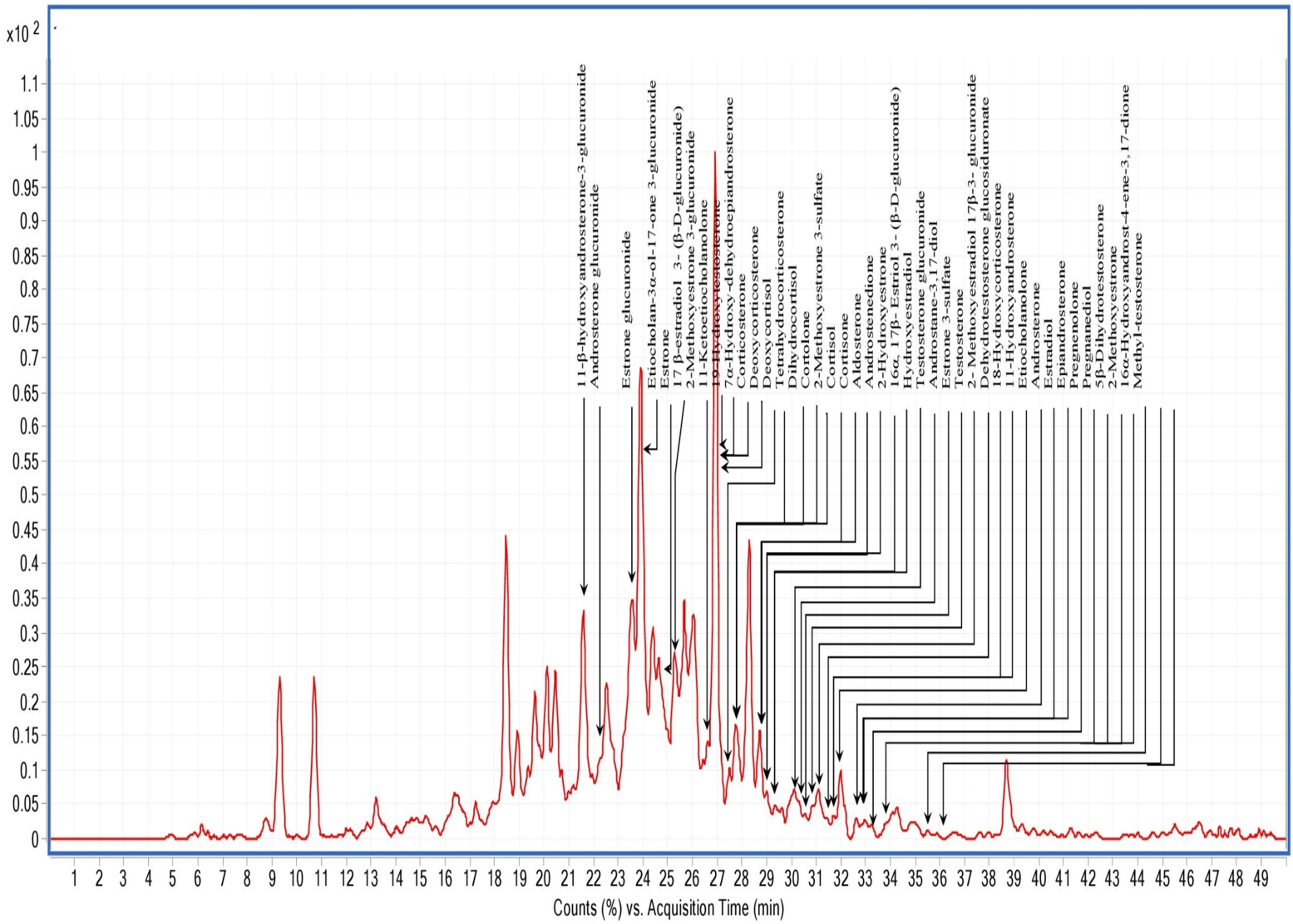

Figure 2: Extracted ion chromatogram showing peaks of different steroids with resolved along time.

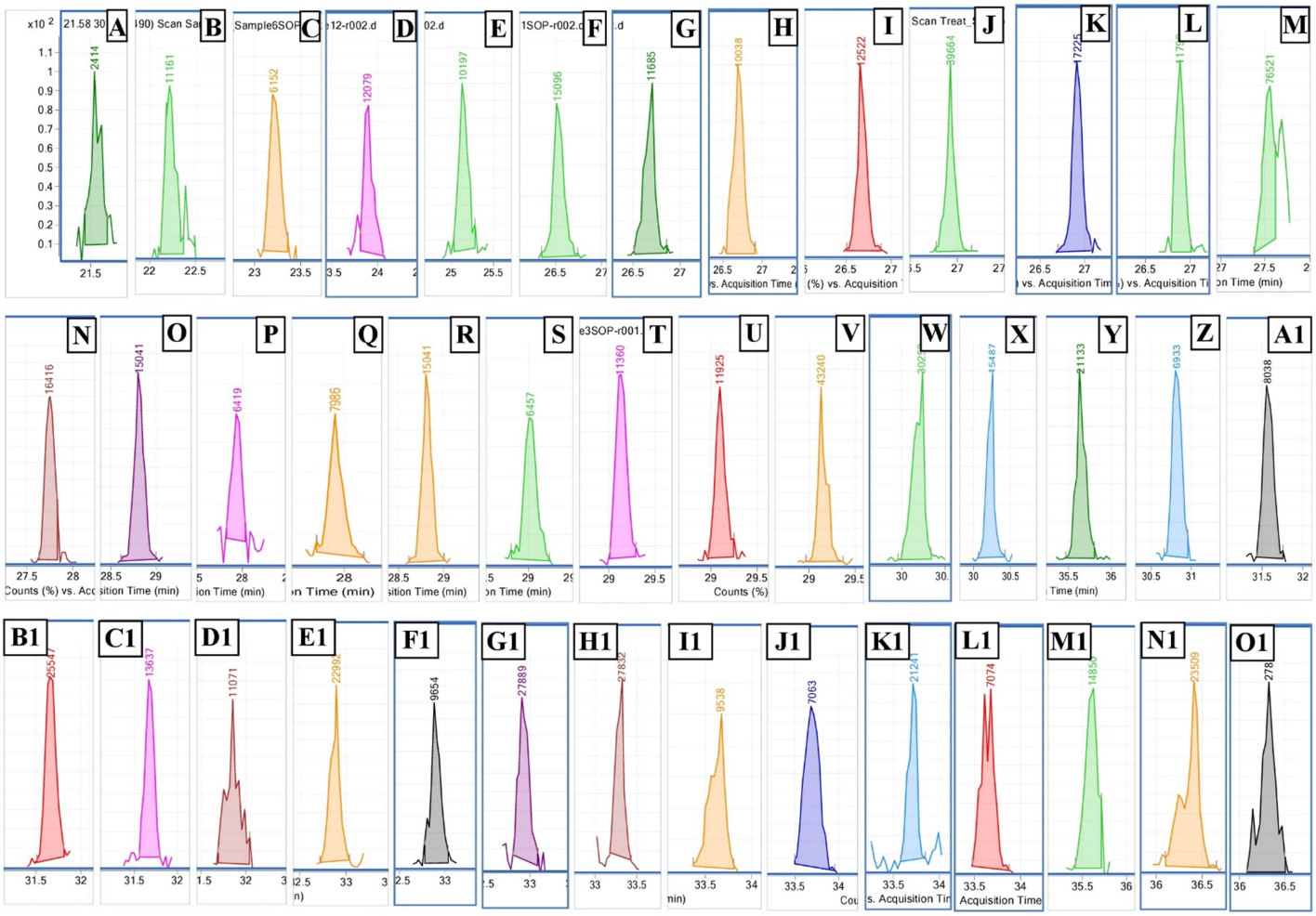

Figure 3: Figure is showing extracted ion peaks with ion abundance. Figure represents (A) 11-β-hydroxyandrosterone-3-glucuronide, (B) androsterone glucuronide, (C) estrone glucuronide, (D) etiocholan-3α-ol-17-one 3-glucuronide, (E) 17 β-estradiol 3- (β-d-glucuronide), (F) 2-methoxyestrone 3-glucuronide, (G) 11-ketoetiocholanolone, (H) 19-hydroxytestosterone, (I) , 7α-hydroxy-dehydroepiandrosterone, (J) corticosterone, (K) tetrahydrocortisone, (L) deoxycorticosterone, (M) deoxycortisol, (N) dihydrocortisol, (O) tetrahydrocortisol , (P) cortolone, (Q) cortisol, (R) cortisone, (S) aldosterone, (T) 2-hydroxyestrone, (U) androstenedione, (V) 16α, 17β- estriol 3- (β-d-glucuronide), (W) hydroxyestradiol glucuronide, (X) testosterone glucuronide, (Y) androstane-3,17-diol, (A1) testosterone, (B1) 6-dehydrotestosterone 17-glucosiduronic acid, (C1) 2- methoxyestradiol 17β-3- glucuronide, (D1) 18-hydroxycorticosterone, (E1) 11-hydroxyandrosterone, (F1) etiocholanolone, (G1) androsterone, (H1) estradiol, (I1) epiandrosterone, (J1) 5β-dihydrotestosterone, (K1) pregnenolone, (L1) pregnanediol, (M1) 2-methoxyestrone, (N1) 16α-hydroxyandrost-4-ene-3,17-dione, and (O1) methyl-testosterone.

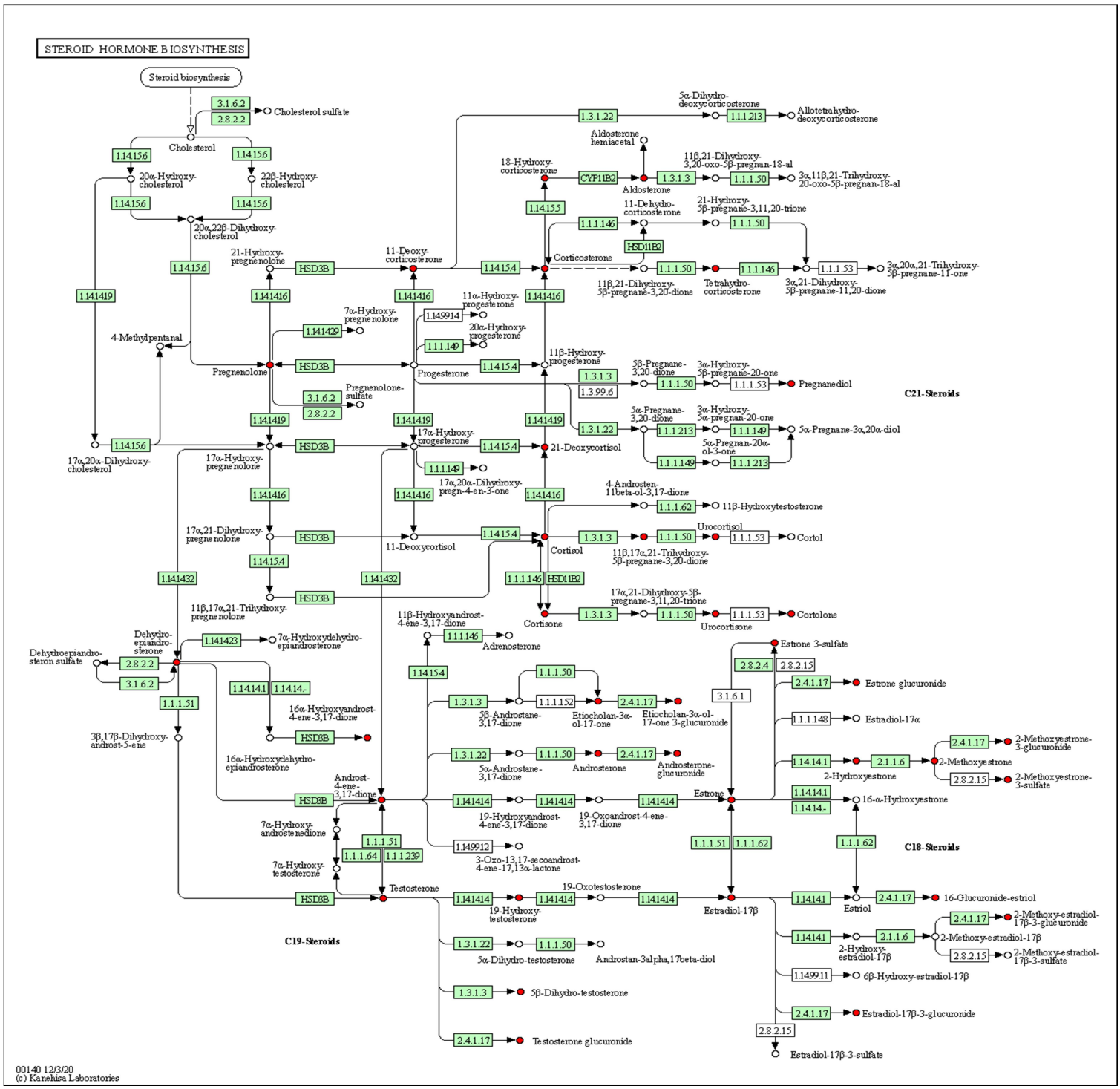

Figure 4: KEGG pathway generated to show the identified urinary metabolite in red dots. Metabolites are related to different step of entire pathway and can be used to predict the effect of metabolite levels changes on the human physiology.