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Metabolite profiling of sex developmental steroid conjugates reveals an association between decreased levels of steroid sulfates and adiposity in obese girls

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ABSTRACT

Free and conjugated steroids coexist in a dynamic equilibrium due to complex biosynthetic and metabolic processes. This may have clinical significance related to various physiological conditions, including sex development involving the reproductive system. Therefore, we performed quantitative profiling of 16 serum steroids conjugated with glucuronic and sulfuric acids using liquid chromatography-mass spectrometry (LC–MS). All steroid conjugates were purified by solid-phase extraction and then separated through a 3-μm particle size C18 column (150 mm × 2.1 mm) at a flow rate of 0.3 mL/min in the negative ionization mode. The LC–MS-based analysis was found to be linear ($r^2 > 0.99$), and all steroid conjugates had a limit-of-quantification (LOQ) of 10 ng/mL, except for cholesterol sulfate and 17β-estradiol-3,17-disulfate (20 ng/mL). The extraction recoveries of all steroid conjugates ranged from 97.9% to 110.7%, while the overall precision (% CV) and accuracy (% bias) ranged from 4.8% to 10.9% and from 94.4% to 112.9% at four different concentrations, respectively. Profiling of steroid conjugates corrected by adiposity revealed decreased levels of steroid sulfates ($P < 0.01$) in overweight and obese girls compared to normal girls. The suggested technique can be used for evaluating metabolic changes in steroid conjugates and for understanding the pathophysiology and relative contributions of adiposity in childhood obesity.

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1. Introduction

Phase II metabolism, also known as conjugation reactions with hydrophilic moieties, is one of the major steroid metabolic pathways, generating biologically inactive metabolites that are excreted via bile or urine. Endogenous steroids are mainly inactivated by sulfation or glucuronidation catalyzed by sulfotransferases (SULTs) or uridine 5′-diphospho-glucuronosyltransferase (UDP-glucuronosyltransferase, UGT) [1–3]. Steroid conjugates with glucurononic and sulfuric acids circulating in the blood can be hydrolyzed back to bioactive, free steroids. Therefore, this metabolism plays an important role in regulating the intracellular levels of total steroids and their biological activities, which may have clinical significance with respect to various diseases and differences of sex development [2–5].

Analysis of steroid conjugates in urine and serum samples using liquid chromatography-mass spectrometry (LC–MS) without hydrolysis and chemical derivatization has been widely used and validated [1,2,4–6]. To reduce the risk of false positives or misleading quantitative results, steroid conjugates of interest should be quantified based on their chemical and physical properties, and with respect to separation efficacies. In contrast to classical bio-analyses that mainly focus on single metabolites and/or defined sets of linked reactions and cycles, the profiling analysis should involve the collection of quantitative signatures on a broad series of metabolites, rather than limited numbers of analytes, to understand metabolic dynamics associated with biological conditions of interest [5–8].

The childhood obesity has renewed interest in the pubertal development [9]. Due to sex developmental metabolism is started from cholesterol, major sex steroids as well as cholesterol,

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pregnenolone and dehydroepiandrosterone (DHEA) were conducted in this study. Here, LC–MS-based quantitative metabolic signatures of steroid conjugates, including 7 glucuronides, 8 sulfates, and 1 glucuronide-sulfate (Table 1), in human serum was therefore developed and validated. Then, the devised analytical method was applied to examine which steroid conjugates showed a change in levels in obese girls as compared with age-matched normal controls. This is because adiposity may alter sex steroids, including DHEA sulfate, in girls [10], and androgen glucuronides could serve as biomarkers of andrognic activity in women [1].

2. Experimental

2.1. Chemicals

The reference standards of the 16 steroid conjugates (Table 1) were purchased from Steraloids (Newport, RI, USA), Sigma (St. Louis, MO, USA), and LGC standards (Teddington, Middlesex, UK). The internal standards, 16,16,17-d_3-testosterone glucuronide for testosterone, dihydrotestosterone, and pregnenolone glucuronides; 16,16,17-d_3-testosterone sulfate for testosterone sulfate; 16,16,17-d_3-dihydrotestosterone sulfate for dihydrotestosterone sulfate; 2,2,3,4,4,6-d_6-DHEA sulfate for DHEA sulfate; 2,4,16,16-d_4-estrone sulfate for estrone sulfate; 16,16,17-d_3-17β-estradiol 3-glucuronide for estrone and 17β-estradiol glucuronides; 2,4,16,16-d_4-17β-estradiol 3-sulfate for 17β-estradiol sulfates; 17α,21,21,21-d_4-pregnenolone sulfate for pregnenolone sulfate; and 25,26,26,26,27,27,27-d_7-cholesterol sulfate for cholesterol sulfate were obtained from LGC standards, Sigma, and C/D/N isotopes (Pointe-Claire, Quebec, Canada).

Sodium acetate (reagent grade), acetic acid (glacial, 99.99%), and formic acid (for mass spectrometry grade, 98% purity) were obtained from Sigma. HPLC-grade methanol and acetonitrile were purchased from Burdick & Jackson (Muskegon, MI, USA). Deionized water was prepared using the Milli-Q purification system (Millipore; Billerica, MA, USA). Ultrafree-MC centrifugal filters (polyvinylidene fluoride, pore size: 0.1 μm; Millipore) were obtained from Millipore.

2.2. Standard solutions and quality-control samples

Stock solutions for all reference standards were prepared at a concentration of 1000 μg/mL in methanol, and all working solutions were made up with methanol at concentrations in the range of 0.1–10 μg/mL. All standard solutions were stored at −20 °C until required. The charcoal-stripped, steroid-depleted processed serum samples (SCIPAC Ltd., Cardiff, UK) were used for the calibration and quality control (QC) samples. QC samples were prepared using steroid-free serum spiked with 16 steroid conjugates at four different concentrations.

2.3. Sample preparation

A solid-phase extraction (SPE) on Strata™-X 33 μm polymeric reversed-phase cartridge (60 mg, 3 mL; Phenomenex, Torrance, CA, USA) was used to extract both steroid glucuronides and sulfates selectively from human serum samples. Before extraction, 0.2 mL of serum samples were spiked with 20 μL of IS mixtures containing 9 deuterated ISs of d_3-TG, d_3-TS, d_3-DHT-S, d_6-DHEA-S, d_4-E1-S, d_3-E2-3G, d_4-E2-3S, and d_4-Preg-S at 1 μg/mL and d_7-chol-S at 100 μg/mL. The samples were diluted with 2.8 mL of 0.2 M sodium acetate buffer (pH 5.2) and vortexed for 30 s to disperse serum proteins. The SPE cartridges were preconditioned with 2 mL of methanol followed by 2 mL of deionized water. After loading the sample onto the Strata-X cartridge, it was washed with 2 mL water and eluted twice with methanol (2 mL). The combined methanol

Table 1
The LC–MS information of 16 steroid conjugates studied.

Compounds	Abbreviation	Molecular weight	Selected ion ($[M-H]^-$,m/z)	Retention time (min)
Reference standards				
Testosterone glucuronide	TG	464.55	463.42	8.68
Testosterone sulfate	TS	368.49	367.33	8.78
Dihydrotestosterone glucuronide	DHT-G	466.56	465.33	9.69
Dihydrotestosterone sulfate	DHT-S	370.50	369.25	9.87
Dehydroepiandrosterone sulfate	DHEA-S	368.49	367.33	9.81
Estrone 3-glucuronide	E1-3G	446.49	445.25	8.46
Estrone 3-sulfate	E1-3S	350.43	349.25	9.38
17β-Estradiol 3-glucuronide	E2-3G	448.51	447.33	7.34
17β-Estradiol 17-glucuronide	E2-17G	448.51	447.33	8.23
17β-Estradiol 3,17-diglucuronide	E2-3G,17G	624.63	623.33	2.92
17β-Estradiol 3-sulfate	E2-3S	352.45	351.25	8.16
17β-Estradiol 3,17-disulfate	E2-3S,17S	432.51	215.22*	5.77
17β-Estradiol 3-sulfate-17-glucuronide	E2-3S,17G	528.57	527.25	5.10
Pregnenolone glucuronide	Preg-G	492.60	491.50	11.12
Pregnenolone sulfate	Preg-S	396.54	395.33	11.90
Cholesterol 3-sulfate	Chol-S	466.72	465.50	29.81
Internal standards				
d_3-Testosterone glucuronide	d_3-TG	467.60	466.42	8.54
d_3-Testosterone sulfate	d_3-TS	371.70	370.33	8.73
d_3-Dihydrotestosterone sulfate	d_3-DHT-S	373.70	372.33	9.75
d_6-Dehydroepiandrosterone-sulfate	d_6-DHEA-S	374.40	373.33	9.69
d_4-Estrone sulfate	d_4-E1-S	354.40	353.33	9.17
d_3-17β-Estradiol 3-glucuronide	d_3-E2-G	451.50	450.33	7.20
d_4-17β-Estradiol 3-sulfate	d_4-E2-S	356.40	355.25	8.08
d_4-Pregnenolone sulfate	d_4-Preg-S	400.50	399.33	11.84
d_7-Cholesterol sulfate	d_7-Chol-S	473.70	472.58	29.72

*$[M-2H]^{2-}$.

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