Journal of Steroid Biochemistry & Molecular Biology 167 (2017) 78–85

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Differences in testosterone and its precursors by sex of the offspring in meconium

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ARTICLE INFO

Article history:
Received 27 July 2016
Received in revised form 7 November 2016
Accepted 14 November 2016
Available online 18 November 2016

Keywords:
Meconium
Prenatal development
Steroids
Androgens
Mass spectrometry
Sexual differentiation

ABSTRACT

Prenatal metabolism exerts profound effects on development. The first stool of the newborn, meconium, provides a window into the prenatal metabolic environment. The objective of this study was to examine the feasibility of meconium as a novel matrix to quantify prenatal steroid levels. We quantified parameters of analytical interest regarding the use of meconium, including sample stability. We hypothesized that meconium steroid content would differ by sex, prompting analysis of meconium to test effects of prenatal steroid metabolism. Meconium from 193 newborns enrolled in the Early Autism Risk Longitudinal Investigation (EARLI) study, including 107 males, and 86 females, were analyzed by isotope dilution-liquid chromatography-high resolution mass spectrometry (ID–LC–HRMS) while blinded to identity for testosterone (T), androstenedione (AD), and dehydroepiandrosterone (DHEA). Steroid levels were compared by sex, and investigations of potential trends resulting from sample storage or processing was conducted. The unconjugated steroid content of meconium in ng/g (mean, standard deviation) was for males: T (2.67, 8.99), AD (20.01, 28.12), DHEA (13.96, 23.57) and for females: T (0.82, 1.63), AD (22.32, 24.38), DHEA (21.06, 43.49). T was higher in meconium from males ($p = 0.0333$), and DHEA was higher in meconium from females ($p = 0.0202$). 6 female and 3 male T values were below the limit of detection. No extreme variability in hydration or trend in steroid levels by storage time was detected. Sexually dimorphic levels of hormones may reflect gestational differentiation, and future studies should consider meconium analysis.

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1. Introduction

Meconium, the first bowel movement of a newborn, is a unique biological sample that provides a window into prenatal development and exposures [1]. The deposition of meconium begins at around 12 weeks of gestation and continues until passed shortly after birth [2]. Molecules in meconium are derived from swallowed or inhaled amniotic fluid, shed epithelial cells, intestinal secretions, and urine. Thus, meconium provides a chemically diverse matrix, potentially reflecting a range of exposures and metabolic processes occurring during gestational development. As all newborns generate significant amounts of this biosample, it may be a source for a wide variety of biomarkers, including biomarkers of exposure, exposure response, and intermediate outcomes relevant to critical developmental processes. Finally, meconium does not require specialized training, equipment, or complicated procedures to collect, thus it can be easily incorporated into epidemiologic studies examining pregnancy and gestation. Such studies are necessary to test the idea that influences in the prenatal period contributes to a wide range of health outcomes from prenatal to adult chronic disease, reflecting influences during gestation as

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http://dx.doi.org/10.1016/j.jsbmb.2016.11.010
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encapsulated by Barker's hypothesis [3] and the concept of Developmental Origins of Health and Disease (DOHaD) [4].

Early development is highly dependent on the endocrine system, and steroid hormones in particular drive development of the fetus [5]. Maternal hormones are required to maintain the pregnancy, but fetal steroids, which are separated from the maternal hormones by the high capacity for steroid metabolism of the placenta, drive sexual differentiation and influence prenatal neurodevelopment. Aberrant prenatal steroid hormone levels are suspected to be linked to a number of adverse health outcomes including disorders of neurodevelopment such as autism spectrum disorder (ASD) [6–9]. Furthermore, many other health outcomes and neurodevelopmental disorders are sexually dimorphic, suggesting that the period of sexual differentiation and steroid hormones may play a role in risk of neurodevelopmental disorders. The period of meconium deposition coincides with sexual differentiation as well as the genesis of neurological structures in the nascent brain [10].

Utilization of meconium in the development of biomarkers is relatively novel compared to other matrices such as urine or blood for which extensive validation, reference ranges, and biomarker studies have been published [11–15]. Previous studies have employed meconium in the examination of prenatal exposures to exogenous compounds including pesticides [16], plasticizers [17], and drugs of abuse including cocaine, alcohol, amphetamines, and nicotine [18–23]. More limited studies have qualitatively described the endogenous contents of meconium to include bile acids and steroid hormones, which indicates that these important classes of molecules may be quantifiable from meconium [2,24,25]. A recent preliminary study investigated biomarkers of gestational diabetes using meconium [26]. Notably, a major benefit of meconium in these studies was that even for a temporally limited exposure, such as drugs of abuse, meconium captured a window of exposure greater than any single comparable biosample such as maternal blood or neonatal urine. Even so, no validated and robust methodology has yet been established for steroid quantitation in meconium. Some cohorts, including the Early Autism Risk Longitudinal Investigation (EARLI), an ASD-enriched risk pregnancy cohort that includes post-delivery biosampling, have taken the step of collecting this matrix along with more commonly utilized biospecimens such as blood and urine from study participants [27].

Measurement of steroid hormones in biosamples is particularly challenging due to their physiochemical properties, biologic potency, and low abundance and thus measurement requires a rigorous approach to provide accurate quantification. We applied isotope dilution liquid chromatography-high resolution mass spectrometry (ID–LC–HRMS) using confirmatory tandem high resolution mass spectrometer (MS/HRMS) [28] to quantify the unconjugated androgen, testosterone (T) and the steroid precursors, androstenedione (AD) and dehydroepiandrosterone (DHEA) from human meconium. This study was designed to test the analytical performance of quantitative measurement of major unconjugated steroid hormones in meconium, and to assess the feasibility and utility of this measurement within a multi-center, longitudinal epidemiological study.

2. Materials and methods

2.1. Study population

EARLI is an enriched autism risk cohort following pregnant mothers with older child diagnosed with ASD (autistic disorder, Asperger syndrome or pervasive developmental disorder not otherwise specified). The design of the EARLI study is described in detail in Newschaffer et al. [27]. EARLI families were recruited at four EARLI Network sites (Drexel/Children's Hospital of Philadelphia; Johns Hopkins/Kennedy Kreiger Institute; University of California: Davis; and Northern California Kaiser) in three distinct US regions (Pennsylvania, Maryland, California). In addition to having a biological child with an ASD confirmed by EARLI study clinicians, eligible mothers also had to communicate in English or Spanish and, at time of recruitment: be 18 years or older; live within 2 h of a study site; and be less than 29 weeks pregnant.

2.2. Biosample collection

Biosamples collection was conducted according to an established EARLI protocol as follows. Mothers were provided with delivery sampling kits for meconium collection prior to delivery. EARLI made arrangements with obstetricians or midwives, and birth hospital labor and delivery staff to assure sample collection and temporary storage. A single meconium sample was collected either at the hospital or birth center. Alternatively, mothers were instructed to "take the sample home as soon as possible and place it in your home freezer". Meconium samples were collected from 193 subjects, with 29 subjects enrolled in the study missing meconium. Meconium collection time was estimated using cord blood collection time (n = 143), or placenta collection time if cord blood collection time was not available (n = 20).

2.3. Steroid measurement

2.3.1. Chemicals

Water, methanol, hexanes, dichloromethane, acetonitrile, and acetic acid were Optima LC–MS grade solvents from Fisher Scientific (Pittsburg, PA). Girard P reagent was from Tokyo Chemical Industry Company, LTD (Tokyo, Japan). Analytical standards for T, AD, and DHEA were from Sigma-Aldrich (St Louis, MO). Stable isotope-labeled steroids ($^{13}C_3$-T, $^{13}C_3$-AD, and $^{2}H_5$-DHEA) were from Cambridge Isotope Labs (Andover, MA).

2.3.2. Dehydration of meconium

Four samples of meconium were randomly selected from each of five groups based upon time of sample collection after birth: 0–2 h, 3–6 h, 7–10 h, 11–17 h, and 18+ hours. For each sample, approximately 20 mg (19.8 ± 2.16) was transferred to pre-massed 1.7 mL snap-cap microcentrifuge tubes. Wet mass for each sample was recorded prior to 24 h of vacuum drying at ambient temperature. Samples were then massed again to obtain dry mass values for each sample. Quantification of meconium hydration was performed by subtraction of the dry mass from the wet mass, then division of the hydration mass by the total wet meconium mass, resulting in percent hydration per sample and per collection time group.

2.3.3. Meconium extraction and analysis of unconjugated steroids

To quantify steroid hormones in meconium, approximately 50 mg (average 50.6 mg) of each of the 193 meconium samples was weighed out into pre-tared 1.5 mL plastic snap cap vials. The wet weight of meconium was recorded to 0.1 mg on a balance with tolerance to +/− 0.01 mg 20 μL of internal standard (10 pg/μL $^{13}C_3$-testosterone, 10 pg/μL $^{13}C_3$-androstenedione, and 50 pg/μL $^{2}H_5$-dehydroepiandrosterone in methanol) were added to each aliquot of meconium. Sample extraction was performed by addition of 1.2 mL methanol, followed by 30 min vortexing and 10 min sonication in a water bath at room temperature. Samples were centrifuged at 10,000 rcf for 10 min, after which methanol was transferred into 15 mL screw cap glass vials. This extraction was then repeated with a fresh 1.2 mL of methanol, and the methanolic extracts for each sample were combined. To confirm that this extraction held at the higher range of steroid content in meconium,

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