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

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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). Steroids 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

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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.

Keywords

meconium; prenatal development; steroids; androgens; mass spectrometry; sexual differentiation

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 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 enriched 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.

Materials and Methods

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 hours of a study site; and be less than 29 weeks pregnant.

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).

Steroid Measurement

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).

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 hours, 3-6 hours, 7-10 hours, 11-17 hours, 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 hours 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.

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 minutes vortexing and 10 minutes sonication in a water bath at room temperature. Samples were centrifuged at 10,000 rcf for 10 minutes, 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, an additional experiment spiking T (1000 ng), AD (1000 ng), and DHEA (5000 ng) into 50 mg of meconium before extraction was conducted.

A 1 mL aliquot of the methanolic meconium extract was transferred to a new 15 mL screw top vial. Next, 1 mL water was added to the unconjugated androgens aliquot followed by addition of 8 mL of 3:2 hexane:dichloromethane. Samples were then vortexed for 10 minutes followed by centrifugation at 2500 rcf for 5 minutes and subsequent freezing at −80°C. The hexane:dichloromethane layer was then decanted off into a new 15 mL screw cap glass vial. Samples were evaporated to dryness under nitrogen, then re-suspended in 200

μL of 10% acetic acid in methanol for derivatization as previously described (29). 20 μL of Girard's reagent P (1 mg/mL in water) was added prior to a 10 minute incubation at 60°C. Finally, samples were again evaporated to dryness under nitrogen before resuspension in 100 μL 50:50 methanol:water. 10 μL of the sample was then used for LC-HRMS analysis.

LC-HRMS analysis on the Girard P derivatives of T, AD, and DHEA were conducted on an Ultimate 3000 quaternary UPLC equipped with a refrigerated autosampler (6° C) and a column heater (60° C) coupled to a Thermo QExactive Plus HRMS as previously described (28). LC separations were conducted on a Phenomenex Kinetix biphenyl column (2.6 μm, 100 A, 100 x 2.1 mm). A multi-step gradient at 0.2 mL/min flow with solvent A (water 1% acetic acid) and solvent B (acetonitrile 1% acetic acid) was as follows; 20% B from 0 to 1 minute, increasing to 25% B from 1 to 5 minutes, increasing to 100% B from 5 to 8 minutes then holding 100% B until 12 minutes, then the column was returned to starting conditions and re-equilibrated at 20% B from 13 to 17 minutes. Column effluent was diverted to waste from 0 to 0.5 minutes and from 14.5 to 17 minutes. The mass spectrometer was operated in positive ion mode with a second generation heated electrospray ionization source (HESI-II) alternating between full scan (200-800 *m/z*) at a resolution of 70,000 and parallel reaction monitoring at 17,500 resolution with a precursor isolation window of 0.7 *m/z*. Source parameters were as follows: capillary temperature, 425 °C, probe temperature, 425 °C, sheath gas, 45 arb units, aux gas, 45 arbitrary units, sweep gas, 2 arbitrary units, spray voltage 4.0 kV, S-lens, 50. Molecular (MH^+) precursor and product ions (*m/z*) used for quantitation were as follows; T and DHEA (422.2802 to 343.2380), AD (420.2646 to 341.2224), $^{13}C_3$-T (425.2903 to 346.2481), $^{13}C_3$-AD (423.2746 to 344.2324), and $^{2}H_5$-DHEA (428.3179 to 349.2757). AD and $^{13}C_3$-AD also manifested a doubly-charged product of double derivatization (2GP-MH^{2+}, 277.1697 m/z for the unlabeled AD). Data analysis was performed in Xcalibur and TraceFinder software (Thermo Fisher, San Jose, CA). Experimenters were blinded to all sample identities until steroid quantification was completed and were then un-blinded to sex and sample collection date.

Analyte stability testing—Twenty 50-mg samples of meconium were taken from a well-homogenized meconium collection and transferred to pre-weighed snap-cap tubes. Five control samples were placed at −80°C for 24 hours, five samples were allowed to sit at room temperature (23-25°C) for 24 hours, while the final pairs of five samples were subjected to 1 and 3 freeze-thaw cycles, respectively. A freeze-thaw cycle consisted of allowing the sample to freeze fully at −80°C for 2 hours prior to allowing the sample to thaw at room temperature for another 2 hours before returning to the freezer. Samples were then extracted, derivatized, and subjected to LC-MS/HRMS analysis as described. These samples were reinjected and analyzed again after one week of storage at 4°C.

To examine the possibility of a chemical instability bias occurring during long-term storage of meconium, levels of each steroid from meconium were examined by time of collection. Due to highly skewed measures, Spearman rank correlation was used to examine any association between each measure (DHEA, T, AD) and sample collection date (in months). A non-parametric test (Kruskal-Wallis test) was performed to see if there was a detectable difference between the means of each measure at three separate year intervals (Dec 2009 - Nov 2010, Dec 2010 - Nov 2011, and Dec 2011 - Nov 2012). One observation with a sample

date outside of these intervals (Nov 2009) was excluded from these analyses. Finally, linear regressions were performed to explore if there was a linear trend between the natural log of each measure and the date of sample collection in months. The natural log was used since the dependent variables (the meconium measures) were highly skewed and had non-normal residuals when looking at regressions on the untransformed meconium measures.

Statistical analysis

Mean, median and IQR were calculated for meconium from males and females. Measures were examined for normal distribution using the univariate SAS skew statistic with manual confirmation by plotting the histogram distributions. All measures were highly skewed | skew| > 0.6. Thus, non-parametric tests or appropriate transformations were performed as indicated in each section. Non-parametric Wilcoxon rank-sum tests were performed to examine any differences between males and females for each meconium steroid measure. Graphing and statistical analysis for method development and quantitation was performed using GraphPad Prism v6 (GraphPad Software, La Jolla, CA), and statistics for EARLI samples were calculated using SAS version 9.3 (SAS Institute, Cary, NC).

Results/Discussion

Variable hydration of meconium

We were unable to detect any significant differences in sample hydration attributable to time after birth at which the meconium was gathered (one-way ANOVA, p = 0.1036) (**Figure 1**). All groups showed an average hydration of 72.98 ± 4.64% (mean ± SD), which is comparable to healthy adult fecal matter, reported at 73-77% (30). The time after birth was estimated from cord blood collection time, or if not available, from placenta collection time, thus this variable may be imprecise. Future work with more controlled conditions in less precious meconium should be conducted to confirm the potential for ambient dehydration or changes by collection time, as this is a concern with quantitation from any fecal matter.

Chromatographic resolution and matrix effects

All steroids were completely resolved by chromatography with retention times of 6.2 min and 8.2 min for the MH^+ of DHEA and T, respectively, and 2.2 min for 2GP-MH^{2+} doubly-derivatized AD which was the predominant form of AD observed (**Figure 2**). Stable isotope analogs co-eluted exactly with the analyte, except for $^2H_5$-DHEA which eluted at 0.1 min earlier than DHEA. Thus, a 13C-labeled analog of DHEA should provide a more suitable internal standard but was not available. T and DHEA were quantified based on the MH^+, and AD was quantified by the more intense 2GP-MH^{2+} form within a 5 ppm window. Confirmatory analysis of all parallel reaction monitoring peaks revealed similar response ratios for analytes across all samples, indicating similar specificity in scan types. Calibration curves were linear for DHEA ($y=0.001386x+0.1028$, $r^2>0.999$) from 16.3 pg on column to 260.4 pg on column, T ($y=0.004744x+0.5009$, $r^2>0.999$) and AD ($y=0.005307x+0.6131$, $r^2>0.997$) from 3.3 pg on column to 52.1 pg on column. In an across-matrix comparison, standard curves constructed in water and charcoal stripped serum produced similar and parallel standard curves indicating no major matrix effects from meconium (**Figure 3**). Inter- and intra-day validation (n=5) gave accuracy and precision below 15% CV except for AD

which was below 25% due to low precision from one run (28). Lower precision for AD is in-line with previously reported findings for this analyte (31). For this study, we defined the limit of detection as the lowest non-zero point of the linear range of the standard curve as the blank samples resulted in no baseline signal for any analyte, further confirming the specificity of this method. LC-HRMS routinely provides high signal to noise (S:N) values, so even in samples near the LoQ the signal to noise remained above 10 (lowest sample quantified S:N of 11 for T). In 9 samples (6 female, 3 male), T was below the limit of quantitation, and thus was estimated as the limit of quantitation/square root of 2. No other analytes were below the limit of quantitation. No certified reference material is available for any of the steroids examined here for meconium, and we did not have sufficient biospecimen to conduct comparisons in this study to RIA or other ligand binding assays for meconium. The assay used has been previously compared to the NIST Standard Reference Material (SRM) for hormones in serum and found to provide values within 5% of the reference method (28).

Quantitative recovery, efficiency of methanol extraction and DCM stripping

Methanolic extraction as employed was efficient in extracting steroids from meconium (**Figure 4**), with a mean (SD) residual 15.2% (1.88%), 12.0% (0.95%), 13.5% (0.49%) of T, AD, and DHEA, respectively, remaining in the re-extracted meconium after an extraction. Commercial charcoal stripped serum was also extracted as a negative control, and meconium soaked in dichloromethane with agitation in a vortexer for an extended period of time (at least 72 hours) removed greater than 90% of steroids from meconium. Recovery from the samples spiked with the higher range of steroids [T (1000 ng), AD (1000 ng), and DHEA (5000 ng)] remained consistent with earlier recoveries at 82.1%, 84.4%, and 87.1% recovery from T, AD, and DHEA, respectively. Use of an appropriate stable isotope labeled internal standard should correct for extraction inefficiency, and further work may continue to optimize recovery procedures from meconium.

Analyte stability over time and temperature variation

No major degradation of analytes was seen as a result of increased exposure to additional freeze/thaw cycles or extended periods of time left at room temperature, with no significant difference in mean values for T, AD, and DHEA between condition groups (**Figure 5**). Higher variability in AD after 24-hours at room temperature may be related to the reported instability of AD (31) or to conversion of other metabolites into AD over time. Due to the longitudinal nature of the study, the meconium was stored before analysis. Using the date of collection, we examined the levels of meconium for trends correlating with storage time. We found no significant correlations with sample collection date (in months) by Spearman rank correlation (r, p-value, DHEA; 0.04, 0.59, T; 0.03, 0.73, AD; −0.02, 0.77) across year intervals by a non-parametric Kruskal-Wallis test (p-value, DHEA; 0.57, T; 0.35 AD; 0.46), or by linear regression of the natural log transformed steroid measures against sample month (p-value, DHEA; 0.21, T; 0.72 AD; 0.62).

Distribution of T, AD, and DHEA by sex

Quantification of T and DHEA in the meconium revealed a striking differential abundance by sex of the newborn (**Table 1**). T was more abundant in meconium from male newborns, with 2.7 (9.1) ng/g [mean, (SD)] wet weight of male's meconium versus 0.83 (1.64) ng/g wet weight of female's meconium ($p = 0.0333$). DHEA displayed the opposite trend, with DHEA of 21.2 (43.7) ng/g wet weight from females and only 13.9 (23.8) ng/g wet weight from males ($p = 0.0202$). We did not observe a difference in AD, 20.2 (28.4) ng/g from males and 22.4 (24.5) ng/g from females.

Discussion

Quantitation of steroids from meconium is possible by LC-MS/MS, even under the conditions, the scale, and the logistics, of epidemiologic pregnancy cohort studies. Meconium from male newborns contained higher T and lower DHEA than the meconium from female newborns while AD levels were comparable across sexes. Controlled experimentation and analysis of stored meconium did not indicate potentially confounding chemical instability in T, AD, or DHEA.

Traditionally, investigations into the molecular influences on fetal development have relied on amniocentesis, placental tissue, cord blood, heel/finger stick blood and urine samples from the newborn to provide unequivocal evidence of fetal exposure as reviewed by Barr (32). These samples all carry significant restrictions as they provide a temporally restricted glimpse into development and the available amount of biosample may be severely limiting. Amniocentesis also introduces the risk of selection bias as it carries a small risk of harm to the fetus and is generally not collected solely for research purposes unless justified with a clinical purpose. Maternal samples, usually blood or urine, only provide maternal exposure, which may be distinct from fetal exposure due to distribution or metabolism.

By comparison, meconium is a promising, easily-obtained biosample that has proven amenable to precise analytical methods. Serving as a repository for toxicants and their metabolites as well as biomarkers of normal and abnormal development, metabolomics analysis of meconium can provide insight into a wide array of exposure, exposure response, and intermediate outcomes as early as 12 weeks in gestation(33). Meconium also serves as a sample of cumulative exposure that may allow a more complete understanding of total fetal internal dose of a given compound. While the majority of meconium analyses in the literature have focused on testing for levels of exogenous chemicals, much potential yet exists for development of assays to quantify targeted endogenous biomarkers. This includes sex hormones studied in this report, as well as strategies to use metabolomic scans of meconium samples in untargeted analyses searching for predictors of disease outcomes of interest as was done in the recent publication on meconium and gestational diabetes (26). The major implication of this work is that future studies investigating prenatal exposures and metabolism should consider biobanking of this sample.

Sex steroids play a multi-faceted role in development, and fetal steroidogenesis is highly orchestrated and organized (5). DHEA is a major circulating steroid precursor, produced by the adrenal glands in humans, whose major form in adult circulation is as a sulfate conjugate

(DHEA-S) which was not examined in this study. Fetal sulfatase activity is thought to be considerably lower than adult (34), thus it is not clear if DHEA-S is a source of fetal steroid precursors. AD is an intermediate in the synthesis of T from DHEA, derived from DHEA by the action of 3-β-hydroxysteroid dehydrogenase (3β-HSD). T is the major male sex hormone in humans; it is metabolized from AD via 17-β-hydroxysteroid dehydrogenase (17β-HSD) or aldo-keto reductase 13C3 (AKR1C3) and modulates development through its action on the androgen receptor. Pathological disorders of steroid metabolism are diverse, owing to the critical reliance of many physiological processes on steroidogenesis and signaling, but the non-fatal dysfunctions of cholesterol and steroid metabolism include many resulting in neurodevelopmental disorders (35). The finding of higher levels of DHEA in the meconium of female newborns may be highly relevant to neurological disorders with prenatal origin as evidence suggests DHEA is neuroprotective and neurostimulatory in a variety of contexts (5). Further, excess DHEA not converted to DHEA-S may result in increased androgen synthesis in females (36).

Measurement of androgens within the prenatal period is of particular importance in testing the androgen hypothesis of autism. This hypothesis posits that the constellation of behaviors typical in ASD are the manifestation of a brain that has been physiologically hyper-masculinized during development (6-9). This increase in fetal androgens may be related to a testosterone-based reduction in expression of the retinoic acid-related orphan receptor-alpha (RORA), a receptor that regulates expression of the aromatase enzyme responsible for converting androgens to estrogens. Protein levels of both RORA and aromatase have been shown to be reduced in the frontal cortex of those with ASD. Further evidence suggests that male hormones negatively regulate expression of RORA and, thus, aromatase, leading to even further elevated levels of androgens (9,37). Measurement of steroid content of meconium offers an avenue to test this hypothesis without the need for amniocentesis. It remains to be determined if the steroid content of meconium reflects external influences (such as exposure to endocrine-disrupting chemicals), and this should be pursued in both observational human and experimental animal studies.

The complexity of meconium may present analytical challenges not addressed here. Radioimmunoassay (RIA) has become increasingly disfavored in steroid measurement due to established concerns about their lack of specificity (38). LC-MS and the related gas chromatography (GC)-MS techniques have replaced RIA as the assays of choice for steroid analysis, despite cost of instrumentation and requirements of technical knowledge (39,40). This shift has mostly stemmed from the lack of specificity in RIA and the increasing sensitivity and performance of LC-MS. Importantly, LC-HRMS provides a sensitive, specific method to quantitate known and identify unknown analytes in a complex matrix (41). Specificity is derived from chromatographic resolution and high resolving power of the MS, which can resolve the isotopic peaks commonly observed in steroid analysis (e.g. the overlap of the second isotopic peak of AD into nominal mass windows of DHEA/T). Instruments capable of tandem or higher order MS (e.g. MS/MS, MS^n) provide additional specificity by selective fragmentation (42). Furthermore, use of MS enables the use of stable isotope labeled analogs of the target analytes to adjust for losses during extraction and analysis, a major consideration in quantitation from a complex biological matrix (43). Finally, to further augment sensitivity, we employed Girard P derivatization for the analysis

of the steroids, which can provide order-of-magnitude increases in sensitivity for steroid metabolites (29).

As this is the first report to our knowledge of measurement of the levels of T, AD, and DHEA in meconium it is difficult to assess our results in a wider context. First, no certified reference material for testosterone is available in meconium as a matrix. Direct comparison to maternal blood would be inappropriate due to the physiology of steroid hormones during pregnancy and the influence of the placenta, and comparison to RIA based amniocentesis levels would likewise be inappropriate due to biospecimen and assay differences. Previous studies in cord blood have shown a sexually dimorphic level of T at 141 and 77 ng/L in males and females, respectively (44). However, AD and DHEA were not investigated in that study. Although not an ideal comparison, reference intervals (in ng/L) by a validated LC-MS/MS method from blood samples are available for Tanner staged pediatric populations at age 6-24 months for T (male <370, female <90), AD (male 25-150, female <150), and DHEA (male <2500, female <1990) (31). Comparison of the relative levels of hormones to each other in our meconium data shows that in contrast to pediatric blood levels, AD and DHEA were at comparable levels (mean 20.2 and 21.2 ng/g) and AD was in fact higher in the pooled meconium samples used for recovery studies. Furthermore, DHEA was higher in meconium from female newborns than male newborns, contrasting to the sex difference seen in blood levels. Future work linking multiple maternal/offspring biospecimens may be highly informative in probing the steroid metabolism of the fetal compartment. Additionally, meconium is known to contain significant amounts of C20, C21, and C22 steroids within the low mg per kg range and further study may quantify the systems level steroid metabolism of the fetus using such high abundance analytes (45-48). A high degree of variation in steroid levels was also observed in the meconium, with relatively large standard deviations and interquartile ranges for all steroids. Further study as to the source of variation in meconium steroid levels is warranted, but evidence here suggests that this is not due to high variability in water content or in storage instability within meconium.

One important caveat of this study is that the population studied is at enriched risk for ASD and neurodevelopmental disorders. Thus, there is a potential for sex differences to be altered by this subject selection. Further validation in a population more generalizable to all births would be appropriate, and a comparison between such a cohort may be informative to the influence of steroid metabolism on risk of neurodevelopmental disorders. Another major limitation of this study is that we did not quantify the estrogens or the conjugated steroids such as DHEA-S. The conjugated steroids may be of particular interest as the role of phase II metabolism including the steroid sulfate and glucuronide conjugates in the fetal unit is poorly understood (34). This also likely explains the relatively low levels of DHEA found in meconium, as DHEA-S is likely the predominant form. Future comparisons to steroid levels in blood suggest that investigating the metabolism of steroid precursors in meconium may reveal differences in fetal versus neonatal and adult steroid levels in the unconjugated and conjugated forms. Further, exposure assessment of sulfated and glucuronidated metabolites may contribute to a greater understanding of fetal metabolism and exposure. Since the estrogens are primarily derived from conversion of androgen precursors, future study into estrogen levels in development may be informative. Studies addressing gestational growth,

especially in the context of environmental exposures and hormonal metabolic influences, should consider collection and analysis of the molecular contents of meconium.

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Highlights

- Measurement of testosterone, DHEA and androstenedione in human meconium
- Testosterone was more abundant in meconium from male newborns.
- Dehydroepiandrosterone was more abundant in meconium from female newborns.

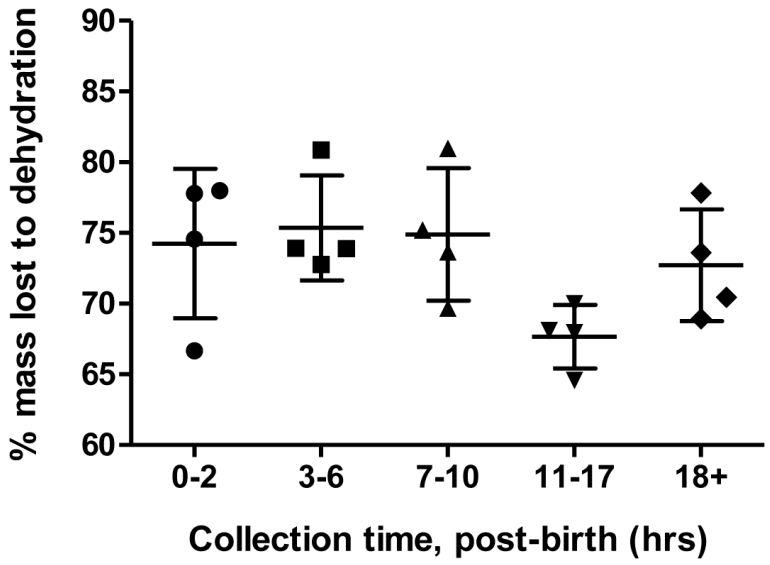

Figure 1.

The hydration of meconium. Vacuum desiccation of meconium gave consistent dehydration. No significant differences found by collection time by one-way ANOVA, or by multiple comparisons.

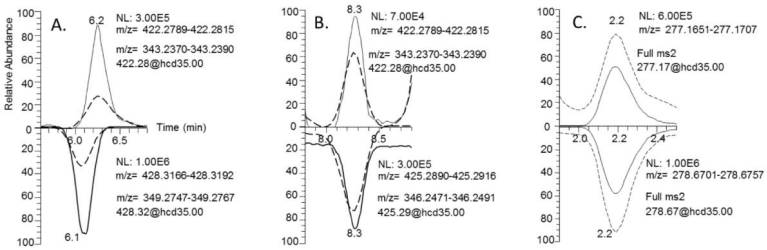

Figure 2.

Representative LC-HRMS and LC-MS/HRMS chromatograms. Chromatograms of steroid analysis from meconium extract for (A) DHEA, (B) T, (C) AD with HRMS (top chromatogram), stable isotope internal standard co-elution (bottom inverted chromatogram), MS/HRMS (dashed lines).

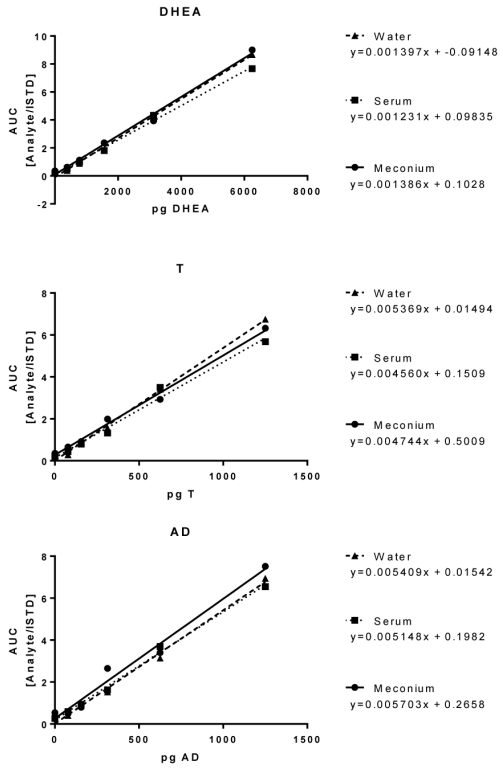

Figure 3.

Standard curves from a comparison of surrogate matrices. Calibration curves prepared with standards spiked into water (triangle, dashed line), charcoal stripped serum (square, dotted line), dichloromethane stripped meconium (circle, solid line) were linear across physiologic ranges for both detected derivatives of DHEA (top), T (middle), and AD (bottom).

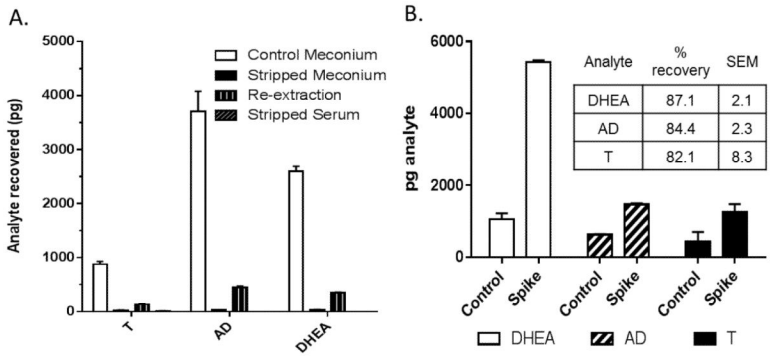

Figure 4.

Recovery of steroids from meconium. (A) Analyte recovery from pooled meconium was tested via extraction from pooled meconium (white), dichloromethane stripped meconium (solid black), re-extraction of pooled meconium (vertical stripe) and as a negative control double charcoal stripped serum (cross-hatch). (B) Quantitative recovery from control meconium spiked with 5000 pg DHEA (white), 1000 pg AD (cross-hatch), and 1000 pg T (solid black). N = 3 for all experimental groups. SEM, standard error of measurement.

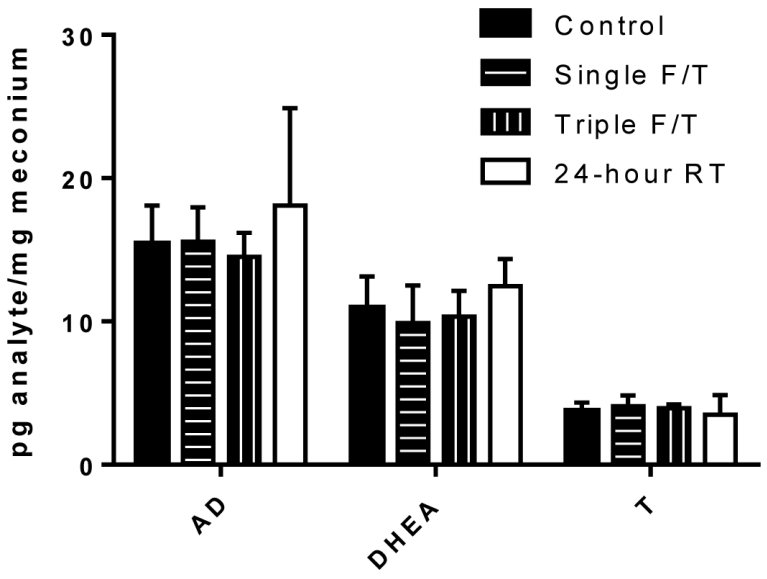

Figure 5.

Steroids are stable in meconium under common storage conditions. Aliquots of homogenized pooled meconium were quantified for AD, DHEA and T content after bench room temperature (RT) storage for 24 hours, and either one (horizontal striped bar) or three (vertical striped bar) freeze/thaw (F/T) cycles as indicated.

Table 1

DHEA, T, AD by sex, standard deviation (SD), and interquartile range (IQR) in EARLI.

Variable	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	p-value^
Free DHEA	21.2 (43.7)	10.4 (7.9, 17.7)	13.9 (23.8)	8.7 (6.5, 12.9)	0.0202
Free Testosterone	0.83 (1.64)	0.21 (0.05, 0.91)	2.7 (9.1)	0.42 (0.06, 1.9)	0.0333
Free AD	22.4 (24.5)	15.1 (12.3, 19.8)	20.2 (28.4)	14.6 (12.6, 16.8)	0.2554