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Abstract

Purpose Tobacco and alcohol use during pregnancy are serious public health concerns and result in adverse developmental outcomes. Identifying in utero exposure is often achieved through meconium analysis or via maternal self-report. In this study, we analyzed fetal liver and placenta to examine second trimester alcohol and smoking exposure.

Methods A validated liquid chromatography-tandem mass spectrometry method for simultaneous analysis of nicotine and its metabolites and alcohol markers (ethyl glucuronide: EtG and ethyl sulfate: EtS) was employed to analyze 193 fetal liver and 48 placenta (*n*=47 paired) samples from electively-terminated pregnancies.

Results EtG, EtS and nicotine markers' limits of detection were 0.7-20 ng/g in fetal samples. Ninety-eight fetal liver and 23 placenta samples were EtG/EtS-positive, while 137 liver and 25 placenta samples were positive for tobacco exposure. When both alcohol markers were present in samples, EtG/EtS ratios were 1.6-11.1 in 17 livers and 2.5-31.1 in 10 placentas. Median [range] summed tobacco marker concentrations were 422 [1.0-2776] and 154 [1.6-1621] ng/g in livers and placentas. Median EtG and nicotine marker concentrations were higher in liver than placenta in paired samples. Strong evidence of exposure occurred in 11 and 22 pairs, respectively, when both samples were positive for alcohol and/or tobacco markers.

Conclusions These paired fetal liver and placenta alcohol and tobacco data provided a unique means for examining the effects of in utero exposure, a critical first step in selecting fetal samples for proteomic and RNA-sequencing studies that could provide mechanisms for adverse developmental outcomes.

Key words Ethyl glucuronide, Nicotine, Fetal liver, Placenta, Pregnancy, Prenatal exposure

Introduction

Tobacco and alcohol use during pregnancy are serious public health concerns, resulting in adverse obstetrical and neonatal outcomes including miscarriage, preeclampsia, low birth weight, preterm delivery, fetal growth restriction, decreased head circumference, placenta abruption, and facial abnormalities [1-4]. Fetal alcohol spectrum disorders (FASD) encompass growth retardation, cognitive impairments, and craniofacial dysmorphology associated with prenatal alcohol exposure [5-6]. Prenatal tobacco exposure also can have a profound impact on infant brain structure and function, infant irritability, and risk for child behavioral and attention disorders [7-8]. Despite these documented health risks, in the 2015 US National Survey of Drug Use and Health (NSDUH), 13.9% of pregnant respondents reported current tobacco use and 9.3% current alcohol use [9].

Identifying prenatal alcohol and tobacco exposure is often achieved through meconium drug quantification and/or maternal self-reported drug use during pregnancy. Meconium is a good matrix for in utero drug exposure assessment in pregnancies carried to term. Although meconium formation begins as early as 13 weeks, other methods must assess earlier exposure. In this study, we examined whether analysis of fetal liver and placenta from electively-terminated pregnancies could determine exposure patterns during the second trimester.

In adults, nicotine is oxidized to cotinine and *trans*-3'-hydroxycotinine (OHCOT) primarily by CYP2A13 and CYP2A6 [10], with additional phase II conjugation to nicotine-*N*-glucuronide (Nic-G), cotinine-*N*-glucuronide (Cot-G), and OHCOT-*O*-glucuronide (OHCOT-G) [10]. Nicotine and these 5 metabolites account for 73-96% of the nicotine dose excreted in adult smokers' urine [10]. Ethyl glucuronide (EtG) and ethyl sulfate (EtS) are chemical markers indicating ethanol ingestion with longer detection times than ethanol. UDP-

glucuronosyltransferase catalyzes ethanol conjugation with glucuronic acid to form EtG [11], while sulfotransferases conjugate ethanol and activated sulfate to form EtS [12]. Fetal liver and placental EtG is likely of maternal origin as EtG readily crosses the placenta and fetal glucuronidation capacity is limited [13-14]. Little is known about placental EtS transfer; however, fetal liver and placental EtS concentrations may result from maternal and/or fetal contributions, as variable yet significant fetal sulfotransferase activity was observed [15-16]. Fetal liver and placental EtG and EtS levels were investigated previously in a small cohort with few positives [17], while there are no available data on human fetal liver nicotine and its metabolites' concentrations.

Adult health is partly programmed during fetal development [18-21]. Gestational exposure to tobacco, alcohol, and other drugs alters normal hormone regulation early in fetal development [22-23]. Mechanisms through which fetal drug exposures result in reduced adult health are poorly understood. We present a validated analytical method for quantifying EtG/EtS and nicotine markers in fetal liver and placenta samples and describe how this method assessed in utero alcohol and tobacco exposure in fetuses from electively-terminated pregnancies. The goal was to select the most appropriate fetal samples to conduct fetal endocrine disruption and DNA/RNA damage assessments.

Materials and methods

Tissue samples

The drug- and alcohol-negative matrix for human fetal liver analyses was a frozen calf liver. Human placentas from healthy pregnancies were donated by volunteers and the Johns Hopkins Bayview Medical Center Pathology Department. Blank matrices were confirmed negative at our

limits of quantification (LOQ) prior to calibrator and quality control (QC) preparation. Samples (193 fetal livers and 48 placentas including 47 paired samples) obtained from women who voluntarily terminated their pregnancies were analyzed. Fetus collection in Aberdeen was approved by the NHS Grampian Research Ethics Committees (REC 04/S0802/21). Women seeking elective terminations of pregnancy were recruited with full written, informed consent by nurses working independently of the study at Aberdeen Pregnancy Counseling Service. Maternal data, medications used, and self-reported number of cigarettes smoked per day were recorded. Only fetuses from normally-progressing pregnancies (determined by ultrasound scan), from women >16 years old and between 8-20 weeks of gestation, were collected following termination as detailed previously [24]. Fetuses were transported to the laboratory within 30 min of delivery, weighed, crown-rump length recorded, and sexed. Collection of human fetal liver and placenta pairs occurred at the University of Newcastle upon Tyne (England) through the Human Developmental Biology Resource at the Institute of Human Genetics under an Institutional Review Board-approved study.

Reagents

S(-)-Nicotine, (±)-nicotine-d_4, (-)-cotinine, (±)-cotinine-d_3, (*R*,*S*)-norcotinine, EtG, EtG-d_5, EtS, and EtS-d_5 were purchased from Cerilliant Corporation (Round Rock, TX, USA); nicotine-*N*-β-D-glucuronide, cotinine-*N*-β-D-glucuronide, cotinine-d_3-*N*-β-D-glucuronide, OHCOT, OHCOT-d_3, OHCOT-*O*-β-D-glucuronide, and (*S*)-cotinine-*N*-oxide as powders from Toronto Research Chemicals Inc. (Toronto, Canada); LCMS grade methanol, ammonium acetate, formic acid, HPLC grade acetonitrile, dichloromethane, isopropanol, ACS grade hydrochloric acid and ammonium hydroxide from Fisher Scientific (Fair Lawn, NJ, USA); fritted filters (10 µm, 15

mL) from United Chemical Technologies Inc. (Bristol, PA, USA); Isolute supported liquid extraction (SLE) columns (1 mg/6 mL) and Evolute-AX anion exchange solid-phase extraction (SPE) cartridges (100 mg/3 mL) from Biotage (Charlotte, NC, USA).

Instrumentation

Tobacco and alcohol markers were quantified on a SCIEX 5500 Qtrap® mass spectrometer with a TurboV electrospray ionization (ESI) source (SCIEX, Foster City, CA, USA), connected to a Shimadzu UFLCXR system with two LC-20ADXR pumps, a CTO-20 AC column oven, and a SIL-20ACXR autosampler (Shimadzu Corporation, Columbia, MD, USA). Data were acquired and processed with Analyst 1.5.1 (SCIEX). A Mini-BeadBeater-8 (BioSpec Products, Bartlesville, OK, USA) pulverized tissue. A CEREX-48 positive-pressure manifold was utilized for SPE (SPEware Corporation, Baldwin Park, CA, USA). Evaporation under nitrogen was conducted in a TurboVap LV evaporator (Zymark, Hopkinton, MA).

Standard Solution Preparation

Individual methanolic standard solutions were diluted to 100 mg/L in methanol. Powdered standards were reconstituted in the manufacturer's recommended solvent and diluted to 100 mg/L in methanol, except for OHCOT, which was maintained at 1 g/L. Methanolic dilutions yielded mixed working calibrator solutions of 0.01, 0.02, 0.05, 0.1, 0.25, 0.5, 1.5 and 3 mg/L for cotinine, and 2.5, 5, 10 and 20 times more concentrated for Nic-G, OHCOT; nicotine, EtS, Cot-G; OHCOT-G; and EtG, respectively. QC solutions were prepared from different stocks than calibrators. Low, medium, and high QCs were prepared across the linear dynamic range for each

analyte. A mixed working internal standard methanolic stock was prepared at 0.1 (cotinine-d_3), 0.25 (EtS-d_5, OHCOT-d_3, Nic-G-d_3), 0.5 (nicotine-d_4, Cot-G-d_3), and 1 mg/L (EtG-d_5).

Procedures

Blank liver or placenta (0.25 g) was fortified with 25 µL calibrator or QC solution or 25 µL methanol for authentic samples, and 25 µL internal standard solution was added. Six to eight 3.2-mm chrome-steel beads were added to each sample, followed by 0.95 mL 0.01% formic acid in methanol and pulverized for 2 s. The methanol was filtered and collected in polypropylene tubes. The original samples were rinsed with an additional 1 mL 0.01% formic acid in methanol, vigorously vortexed for 1 min, and filtered into the same tubes. Two 450-µL aliquots were transferred to separate tubes for SLE of nicotine and its metabolites and for anion-exchange SPE of EtG and EtS.

For nicotine and metabolite extraction, 525 µL 0.25% ammonium hydroxide in methanol was added to the nicotine aliquot yielding pH >10. Samples were vortexed and poured onto 1 mL SLE beds. After 5 min equilibration, analytes were eluted with 2 x 2.5 mL 95:5 dichloromethane/isopropanol into glass centrifuge tubes. Positive pressure was gradually applied up to 2.4 L/min to complete elution. A 50-µL volume of 1% HCl in methanol was added to the eluents before evaporation under nitrogen at 35°C. Reconstitution occurred in 200 µL of 10 mM ammonium acetate in water with 5 µL methanol added to aid pellet formation. Samples were vortexed and centrifuged at 1800*g* at 4°C for 5 min. A 150-µL volume was transferred to a microcentrifuge tube and centrifuged at 20,800*g* for 5 min at 4°C. A 125-µL volume was transferred to autosampler vials and 5 µL injected. For EtG and EtS extraction, 2 mL acetonitrile

and 10 µL 236 mM ammonium hydroxide were added to the 450 µL tissue supernatant, and the SPE followed our published meconium assay for these markers [25].

Liquid chromatography-tandem mass spectrometry

Nicotine and its metabolites were chromatographically separated on a Poroshell 120 EC-C8 column (150 x 2.1 mm i.d., 2.7 µm particle size), fitted with a matching guard column (Agilent Technologies, Santa Clara, CA, USA) similarly to a previous publication [26]. Mobile phases A and B were 10 mM ammonium acetate in water and methanol, respectively. At 0.3 mL/min flow, the gradient started at 0% B, increased to 60% B over 6 min, increased to 100% B in 0.1 min, held at 100% B for 2.4 min, decreased to 0% B in 0.1 min, and held for 2.9 min; total run time was 11.5 min. Liquid chromatograph (LC) eluent was diverted to waste for the first min and the final 5.5 min.

Mass spectrometric data for nicotine and metabolites were acquired via positive ESI. Compound-specific tandem mass spectrometry (MS/MS) parameters were optimized by infusing 10 µg/L reference solutions at 10 µL/min dissolved in A/B (50:50, v/v) mobile phase (Table S1). Optimized source parameters were gas 1 60 psi, gas 2 40 psi, curtain gas 45 psi, source temperature 700°C, and ion spray voltage 5500 V. Three periods were utilized to acquire multiple reaction monitoring (MRM) mode data with period 1 (2.5 min, 100 ms dwell) consisting of Cot-G and Cot-G-d_3; period 2 (1.7 min, 50 ms dwell) Nic-G, OHCOT-G, and Nic-G-d_3; and period 3 (7.3 min, 30 ms dwell) OHCOT, cotinine, nicotine, OHCOT-d_3, cotinine-d_3, and nicotine-d_4. Additional MRMs for two isobaric compounds were not quantified; cotinine-*N*-oxide (*m/z* 193.0>96.0, 193.0>78.8) and norcotinine (*m/z* 163.0>80.0, 163.0>118.1) were only monitored in periods 2 and 3, respectively.

LC and MS parameters for EtG and EtS were adapted from our previously published meconium method [25]. However, the quantifying EtG transition, *m/z* 221>75, was replaced by *m/z* 221>85m/z, due to matrix interferences at low concentrations (Table S1).

Validation

Sensitivity, linearity, specificity, accuracy, imprecision, extraction efficiency, matrix effect, dilution integrity, carryover, and stability were evaluated according to Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines [27] as summarized in Table S2.

Results

This method is the first to offer simultaneous extraction of EtG, EtS, nicotine, cotinine, OHCOT, and 3 prominent nicotine glucuronide metabolites from a human fetal liver or placenta sample. Although initial tissue extraction was simultaneous, marked physiochemical differences between acidic EtG and EtS and basic nicotine and metabolites required different SPE and LC approaches.

Limits of detection (LODs), LOQs, linear ranges, and calibration curves for both matrices are presented in Table 1. Accuracy and imprecision results for liver and placenta are presented in Table 2. Extraction recoveries and matrix effects for liver and placenta are given in Table 3. Stability results for all conditions and all samples are summarized in Table 4. All validation experiments met acceptability criteria (Table S2).

The validated methods were used for the analysis of 193 fetal livers and 48 placentas (47 paired) samples. Summarized concentration data for all analytes and matrices are found in Table 5. When both alcohol markers were present in a sample (17 livers and 10 placenta samples), an

EtG/EtS ratio was calculated and ranged from 1.6-11.1 in liver and 2.5-31.1 in placenta. The six tobacco marker concentrations in each individual sample were summed. Median [range] summation concentrations were 422 [1.0-2776] and 154 [1.6-1621] ng/g in liver and placenta, respectively.

We identified in utero alcohol and tobacco exposures based on EtG/EtS and nicotine marker concentrations in liver and placenta. Currently, there are not sufficient pre-existing fetal data in the literature to guide interpretation. Our criteria for determining evidence of alcohol and tobacco exposure are described in Table 6, based on presence of and concentrations of multiple analytes. For each matrix, numbers of samples in each predicted exposure category are also tabulated. Results from this study are utilized for selecting fetuses for later DNA and RNA analyses that will examine exposure and possible damage at the genomic level.

When both alcohol markers were detected together, EtG concentrations were 27.0-2050 ng/g and 34.3-1168 ng/g, and EtS concentrations were 7.3-423 ng/g and 7.9-214 ng/g in 17 fetal livers and 10 placentas, respectively. Only one fetal liver had detectable EtG (31.9 ng/g) without EtS with no paired placenta for comparison, while 6 placenta samples were positive for EtG only (35.1-628 ng/g). EtS was often detected alone in 81/193 fetal liver (5.1-42.0 ng/g) and 7/48 placenta (6.9-35.6 ng/g) samples. Median EtG concentrations were higher in liver than placenta (Table 5). Of the 11 paired samples indicating strong evidence of recent alcohol exposure, 3 pairs contained both markers in both matrices, while 3 pairs were negative in liver but positive for EtG and EtS in placenta. Overall, 95/193 fetal liver and 25/48 placenta samples, including 21 paired samples, were negative for EtG and EtS (Table 6).

One or more nicotine markers were detected in 137/193 liver and 25/48 placenta samples. Median nicotine and metabolite concentrations were higher in liver than in placenta, including

median concentration sums (Table 5). Low tobacco marker concentrations or presence of few analytes were difficult to interpret and were classified as weak or moderate evidence of recent exposure, but did not preclude previous maternal use if there was a period of abstinence prior to pregnancy termination. Summed nicotine marker concentrations weakly indicative of recent tobacco exposure were 1.0-13.0 ng/g and 1.6-10.8 ng/g in fetal liver and placenta, respectively, while moderate evidence encompassed summed concentrations of 8.3-421 ng/g and 43.7-154 ng/g in fetal liver and placenta, respectively. When strong evidence of tobacco exposure was predicted, summed concentrations were 290-2776 ng/g and 148-1621 ng/g in fetal liver and placenta, respectively (Table 5). Of the paired samples, 6 indicated strong evidence of both alcohol and tobacco exposure. No tobacco analytes were detected above the LOQ in 56/193 fetal livers and 23/48 placentas, including 20 paired samples (Table 6).

Discussion

This novel, comprehensive method was fully validated according to SWGTOX guidelines [27] for EtG, EtS, nicotine and phase I and phase II metabolites in a fetal liver or placenta sample. The methodology was successfully applied to the analysis of 193 fetal liver and 48 placenta samples from electively terminated pregnancies for the determination of in utero exposure to alcohol and tobacco.

Our investigation is only the second to look at fetal liver and placental EtG and EtS. A previous study by Morini et al. [17] tested 35 matched liver-placenta pairs from women who terminated their pregnancy at week 12. In this previous study, four fetal livers were EtG-positive (33-391 ng/g) with three also positive for EtS (15-51 ng/g). In placenta, four were EtG- (122-1307 ng/g) and EtS- (10-126 ng/g) positive and were paired with the four fetal livers with

detectable EtG. Two additional placenta samples were positive only for EtS (29 and 175 ng/g). EtG/EtS ratios in the three dual positive liver samples were 3.9, 5.3, and 7.3; while EtG/EtS ratios in the four dual positive placentas were 12.7, 12.7, 4.8, and 10.4. EtG and EtS were always higher in placenta compared to those of liver [17]. Our EtS LOQ is the same as that of Morini et al. (5 ng/g) [17], but our EtG LOQ was 20 ng/g. We fully homogenized the 250 mg liver by bead-beating, enabling a more efficient and reproducible release of drugs as compared to an acetonitrile wash reported by them, but produced additional matrix effects, requiring us to raise our LOQ. However, our LOQ was below the concentrations in all samples identified and reported by them.

In order to select samples for further testing, we examined alcohol and tobacco markers in fetal, infant, and adult studies. EtG readily crosses the placenta [20]; therefore, fetal liver EtG is likely to be primarily of maternal origin, as fetal glucuronidation capacity is limited [13]. No placental diffusion EtS studies exist. However, fetal liver sulfotransferases showed variable yet significant activities [15]. It is possible that the fetal liver EtS is of fetal origin, or of both the fetal and maternal origin.

When considering adult alcohol markers, if EtG and EtS are present, there is a strong indication of alcohol ingestion [28]. EtG and EtS are only produced by antemortem drinking, enabling differentiation of postmortem ethanol formation from antemortem drinking. EtG was reported to occasionally degrade in postmortem samples [28-29], suggesting superiority of EtS analysis. In addition, EtS is not formed in the presence of putrefaction or with lack of preservatives [28,30]. For our fetal samples, presence of EtS or EtG only as well as that of both compounds can be considered alcohol-exposed.

In meconium, we previously reported that COT, NIC, or OHCOT concentrations ≥10 ng/g suggested active maternal tobacco exposure during pregnancy [31], although subsequent work by our group found lower cutoffs, equivalent to analytical LOQs (1 ng/g COT, 2.5 ng/g NIC, and 5 ng/g OHCOT), and better identified active maternal smoking [3]. This suggests that detecting any marker in meconium indicates maternal smoking during pregnancy, and environmental or passive exposure would produce undetectable tobacco marker concentrations in meconium. In serum, COT ≤3 ng/mL differentiated passive from active maternal exposure [10,31]. In support of our previous 10 ng/g cutoff, Braun et al. [32] showed that in infants of tobacco-smoking mothers with a mean serum COT >3 ng/mL, the lower limit of the 95% confidence interval for meconium COT was 10 ng/g. Additionally, among these infants with exposure to maternal active tobacco smoking, multiple meconium tobacco markers were detected [31]. For our fetal samples, presence of multiple markers, especially above the median, was considered the criteria for tobacco-exposed.

To our knowledge, these are the first fetal liver tobacco marker concentrations, although there are other placenta and fetal human brain concentration data. The transfer rate for nicotine across the placenta was about 90% [33]. In 12^{th} week-terminated pregnancies, evidence of fetal tobacco exposure was observed, with placenta median (range) COT and NIC concentrations of 80 (25-190) ng/g and 61 (33-120) ng/g, respectively [34]. In a single human postmortem fetal brain sample, 40 ng/g COT, 65 ng/g OHCOT, and no NIC were detected [35], although the timing of tobacco exposure was unavailable. In adult postmortem liver, COT was always higher (4-25 times) than NIC and both analytes were detected at higher concentrations than in blood; COT and NIC concentrations in adult liver were 260-1586 ng/g and 14-325 ng/g, respectively [36].

We predicted alcohol and tobacco exposure based on analyte presence and their concentrations, although there are few data to guide interpretation. It is important to publish quantitative data for future investigations. Molecular analyses in prenatal alcohol and tobacco-exposed fetuses may eventually suggest mechanisms for adverse fetal outcomes and perhaps correlations between fetal liver or placenta concentrations and outcomes. Relevantly, a balanced population of the 80 fetal livers and the 145 Aberdeen fetal livers that we report here is currently being analyzed by shotgun proteomics and RNA-sequencing. No recent alcohol exposure was demonstrated by absence of EtG and EtS, as absence of these markers may only indicate maternal abstinence for a few days prior to termination. It is possible that these samples could provide a wider window of alcohol or tobacco detection, but we have no data on timing or magnitude of exposure. The fetal liver EtG detection window following acute or chronic maternal alcohol consumption is unknown, although EtG and EtS are detected in adult urine for 40-130 h after the end of drinking in most cases [37-38].

Fetuses exhibiting strong evidence of recent alcohol exposure were selected primarily from samples positive for both EtG and EtS (n = 17 for liver, n = 10 for placenta), with an additional 2 placenta identified when EtG concentration ≥ median concentration. Moderate evidence of recent alcohol exposure identified 45 liver and 4 placenta samples with EtS detected ≥ median concentration despite no detectable EtG. Given potential EtG instability and higher EtG LOQ, EtS-only positive cases could indicate exposure. In addition, differential metabolic pathway rates in mothers or fetuses could result in more EtS than EtG as EtS is stable [28], and there are no published cases of artificial EtS formation. However, in adults, urine EtS detection could occur after extreme non-traditional drinking behaviors, including non-alcoholic beer [39], sauerkraut [39], ripened bananas [39], grape juice [39], and non-alcoholic wine [29]. In the

present study, three placentas were EtG and EtS positive, although paired liver samples were negative. Additionally, three liver samples were positive for EtS-only (8.6, 9.9, and 17.0 ng/g) with no analytes detected in placenta, while nine EtS-only placenta (6.9-35.6 ng/g) samples had paired liver samples negative for both alcohol markers. Therefore, alcohol exposure was suggested in this study, but cannot be definitively determined.

No recent tobacco exposure was identified in fetal liver and placenta samples that were negative for all six tobacco markers. Tobacco marker absence may indicate recent maternal abstinence but not necessarily throughout pregnancy. Detection windows for tobacco markers in fetal liver and placenta are unknown. In cases where only a few markers were detected, interpretation was difficult and low concentrations could possibly be attributed to minimal maternal smoking, abstinence for a few days prior to termination or passive maternal tobacco smoke exposure. Large population sampling studies showed positive, but low, mean serum cotinine concentrations among self-reported nonsmokers and passively exposed individuals [10]. For this reason, samples positive for 5/6 or 6/6 analytes with high summed concentrations were considered to exhibit strong evidence of recent tobacco exposure, especially due to the presence of phase I and phase II nicotine metabolites.

Conclusions

While timing of gestational alcohol or tobacco use is unknown, we were able to identify multiple, specific alcohol and tobacco markers for selecting samples for proteomic and RNA-sequencing studies. Our validated analytical method successfully quantified alcohol (EtG and EtS) and tobacco markers (nicotine and phase I and phase II metabolites) and sought to predict alcohol and tobacco exposure. After consulting the literature for fetal studies and examining

analyte concentrations in other matrices, we stratified our fetal liver and placenta data to categorize strength of evidence of recent maternal alcohol or tobacco consumption, exposing pre-term fetuses to potentially harmful chemicals at a young gestational age. For the first time, a large cohort of paired, exposed fetal liver and placenta samples were analyzed. Publishing these data is crucial to inform future research, as this is the first comprehensive analysis of paired fetal liver and placenta samples for evidence of in utero alcohol and tobacco exposure.

Acknowledgements

This research was supported by the Medical Research Council (UK) grant MR/L01 0011/1 and the Intramural Research Program at the National Institute on Drug Abuse of the National Institutes of Health. Paired fetal liver and placenta samples were graciously provided by the Joint Medical Research Council/Wellcome Trust (grant number 099175/Z/12/Z) Human Developmental Biology Resource (www.hdbr.org).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of all involved institutions and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Collection of fetal liver material was approved by the National Health Service Grampian Research Ethics Committees (REC04/S0802/21). This article does not contain any studies with animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study. No identifying information is included in this article.

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Table 1 Linearity, and limit of detection (LOD) and limit of quantification (LOQ) results

Analyte	LOD (ng/g)	LOQ (ng/g)	Linear range (ng/g)	Liver			Placenta		
				y-Intercept (mean±SD, *n*=5)	Slope (mean±SD, *n*=5)	R^2 (range, *n*=5)	y-Intercept (mean±SD, *n*=5)	Slope (mean±SD, *n*=5)	R^2 (range, *n*=5)
Cotinine-*N*-glucuronide	5	5	5-1500	0.011 ± 0.065	1.223 ± 0.128	0.997 - 0.999	-0.024 ± 0.018	1.252 ± 0.054	0.993 - 0.999
Nicotine-*N*-glucuronide	1.75	2.5	2.5-750	-0.025 ± 0.005	0.812 ± 0.060	0.993 - 0.998	-0.029 ± 0.004	0.832 ± 0.025	0.994 - 0.998
OHCOT-*O*-glucuronide	7	10	10-1500	0.000 ± 0.000	0.017 ± 0.001	0.995 - 1.000	-0.001 ± 0.001	0.034 ± 0.007	0.992 - 1.000
OHCOT	1.75	2.5	2.5-750	-0.005 ± 0.011	1.172 ± 0.027	0.997 - 0.999	-0.003 ± 0.006	1.134 ± 0.018	0.998 - 0.999
Cotinine	0.7	1	1-300	0.040 ± 0.019	0.982 ± 0.039	0.993 - 0.998	0.054 ± 0.016	1.007 ± 0.065	0.996 - 0.999
Nicotine	3.5	5	5-1500	0.066 ± 0.044	1.058 ± 0.024	0.995 - 0.998	0.085 ± 0.047	1.172 ± 0.218	0.993 - 0.998
Ethyl sulfate (EtS)	5	5	5-1500	0.014 ± 0.031	1.200 ± 0.010	0.997 - 0.999	0.000 ± 0.000	1.100 ± 0.032	0.996 - 0.999
Ethyl glucuronide (EtG)	20	20	20-3000	-0.050 ± 0.111	7.182 ± 0.270	0.989 - 0.998	0.000 ± 0.000	5.036 ± 0.282	0.996 - 1.000

SD standard deviation, *OHCOT trans*-3'-hydroxycotinine

Table 2 Accuracy (bias) and imprecision results for tobacco and alcohol markers in fetal liver and placenta samples

Analyte	Concentration (ng/g)	Liver				Placenta			
		Mean between-run bias (%, *n*=20)	Within-run bias range (%, *n*=5)	Between-run imprecision (%CV, *n*=20)	Maximum-within run imprecision (%CV, *n*=5)	Mean between-run bias (%, *n*=20)	Within-run bias range (%, *n*=5)	Between-run imprecision (%CV, *n*=20)	Maximum-within run imprecision (%CV, *n*=5)
Cotinine-*N*-glucuronide	15	99.1	88.7 - 109	5.2	9.9	96.6	84.0 - 117	11.9	13.2
	300	104	91.7 - 114	5.4	6.1	102	91.7 - 113	5.7	5.9
	1200	101	90.8 - 114	5.5	5.9	108	92.5 - 116	5.6	7.8
Nicotine-*N*-glucuronide	7.5	106	94.8 - 113	4.2	4.6	99.7	94.5 - 112	4.5	5.9
	150	97.6	87.3 - 112	6.6	6.7	94.9	84.7 - 108	5.4	9.1
	600	92.4	84.5 - 103	6.9	6.7	99.5	87.2 - 112	7.5	7.4
OHCOT-*O*-glucuronide	30	106	94.0 - 115	5.8	6.4	105	89.0 - 119	8.5	6.9
	600	104	92.3 - 115	6.7	7.8	104	92.3 - 119	8.6	10.6
	1200	108	97.5 - 115	5.5	5.8	107	90.8 - 116	7.4	10.1
OHCOT	7.5	110	102 - 115	3.3	6.0	113	105 - 117	2.4	4.7
	150	110	106 - 115	2.4	3.5	113	103 - 117	3.6	6.9
	600	107	94.5 - 115	4.7	4.0	110	99.7 - 115	3.6	6.0
Cotinine	3	102	92.3 - 113	5.9	7.1	106	97.7 - 114	4.1	3.5
	60	104	94.8 - 113	5.3	6.6	107	92.2 - 115	5.0	8.6
	240	95.5	87.9 - 113	8.3	7.7	94.9	85.0 - 107	6.2	8.2
Nicotine	15	107	88.7 - 115	6.9	12.1	107	90.7 - 117	7.3	10.6
	300	107	99.3 - 115	4.7	4.5	108	95.0 - 115	5.0	7.8
	1200	111	99.2 - 115	3.5	4.9	108	80.7 - 116	10.6	13.0
Ethyl sulfate (EtS)	15	105	95.3 - 115	5.9	5.8	108	98.7 - 117	4.7	6.9
	300	103	95.7 - 115	5.0	4.7	107	92.3 - 116	5.2	8.3
	1200	106	98.3 - 114	4.0	3.6	106	93.3 - 115	5.2	4.8
Ethyl glucuronide (EtG)	60	98.3	86.8 - 113	8.6	10.4	98.4	85.3 - 107	4.7	8.5
	1200	93.9	84.2 - 107	8.8	10.3	97.5	85.0 - 108	7.3	8.2
	2400	105	87.9 - 115	8.4	9.6	110	90.8 - 118	5.6	8.8

CV coefficient of variation

Table 3 Extraction efficiencies and matrix effects for tobacco and alcohol markers in liver and placenta samples

Analyte	Liver				Placenta			
	Extraction efficiency (%, *n*=5)		Matrix effect (%, *n*=5)		Extraction efficiency (%, *n*=5)		Matrix effect (%, *n*=5)	
	Low[a]	High[b]	Low	High	Low	High	Low	High
Cotinine-*N*-glucuronide	54.4	53.0	12.3	11.0	31.6	31.3	-16.6	-32.4
Nicotine-*N*-glucuronide	64.4	70.5	-40.0	-25.2	48.8	56.6	-2.5	-16.3
OHCOT-*O*-glucuronide	60.4	57.9	-57.3	-50.3	55.9	60.9	-15.6	-35.1
OHCOT	92.6	93.6	-44.3	-32.4	88.5	93.9	-11.2	-20.8
Cotinine	92.3	92.2	-42.2	-32.8	107	103	-22.7	-23.9
Nicotine	90.9	80.3	-33.2	-33.3	82.8	92.2	8.0	-21.3
Ethyl sulfate (EtS)	76.1	71.9	-30.6	-17.5	73.1	68.1	-24.5	-10.5
Ethyl glucuronide (EtG)	86.8	86.0	-50.6	-51.4	94.1	91.5	-27.3	-24.5
Cot-G-d_3	49.2	45.8	17.3	14.8	27.6	32.4	-10.0	-27.1
Nic-G-d_3	57.9	59.7	-38.2	-25.7	51.4	58.2	-4.4	-8.3
OHCOT-d_3	85.7	82.7	-43.4	-28.6	91.3	89.7	-18.2	-14.2
Cotinine-d_3	89.1	83.6	-45.7	-32.1	102	101	-35.8	-23.7
Nicotine-d_4	80.4	73.9	-42.5	-27.9	93.4	92.1	-18.8	-16.3
EtS-d_5	63.4	72.6	-34.9	-35.9	68.0	74.0	-28.6	-38.3
EtG-d_5	78.1	96.5	-44.1	-50.5	88.3	97.1	0.2	-11.4

Cot-G cotinine-*N*-glucuronide, *Nic-G* nicotine-*N*-glucuronide

[a]Low quality control concentrations were 3 ng/g cotinine, 7.5 ng/g nicotine-*N*-glucuronide and OHCOT, 15 ng/g nicotine, cotinine-*N*-glucuronide, and EtS, 30 ng/g OHCOT-*O*-glucuronide and 60 ng/g EtG

[b]High quality control concentrations were 240 ng/g cotinine, 600 ng/g nicotine-*N*-glucuronide and OHCOT, 1200 ng/g nicotine, cotinine-*N*-glucuronide, EtS and OHCOT-*O*-glucuronide, and 4800 ng/g EtG

Table 4 Stability results (%, *n* = 4) of fortified liver and placenta samples in various storage conditions prior to and following extraction

Analyte	12h Room temperature		72h 4°C		3 Freeze/thaw cycles		72h Autosampler (4°C)		5 Days supernatant[c] (4°C)	
	Low[a]	High[b]	Low	High	Low	High	Low	High	Low	High
Liver										
Cotinine-*N*-glucuronide	84.4	83.3	87.6	101	96.9	96.7	98.8	99.6	96.4	92.6
Nicotine-*N*-glucuronide	86.1	83.0	95.8	93.8	89.2	84.6	97.4	85.6	90.8	81.4
OHCOT-*O*-glucuronide	89.1	101	109	117	94.4	111	111	106	108	104
OHCOT	114	109	110	116	110	110	111	101	107	94.3
Cotinine	112	115	113	108	113	98.5	104	94.9	98.6	85.6
Nicotine	110	113	112	113	115	108	112	99.3	104	92.3
Ethyl sulfate (EtS)	106	103	101	105	103	105	102	111	104	107
Ethyl glucuronide (EtG)	91.1	106	98.1	99.7	102	104	97.0	109	95.2	98.2
Placenta										
Cotinine-*N*-glucuronide	106	108	91.3	99.1	101	107	88.3	116	98.7	113
Nicotine-*N*-glucuronide	97.3	103	91.8	87.0	103	90.6	97.4	104	98.5	93.2
OHCOT-*O*-glucuronide	115	116	96.3	92.2	99.5	96.0	109	114	97.0	112
OHCOT	115	108	110	102	116	111	115	112	115	111
Cotinine	88.3	81.7	110	96.2	111	98.4	108	101	104	94.7
Nicotine	81.9	86.4	111	106	109	112	99.1	90.4	109	117
Ethyl sulfate (EtS)	107	97.3	99.9	103	103	113	108	104	107	113
Ethyl glucuronide (EtG)	96.5	104	92.6	95.1	101	113	113	116	102	113

[a]Low quality control concentrations were 3 ng/g cotinine, 7.5 ng/g nicotine-*N*-glucuronide and OHCOT, 15 ng/g nicotine, cotinine-*N*-glucuronide, and EtS, 30 ng/g OHCOT-*O*-glucuronide and 60 ng/g EtG

[b]High quality control concentrations were 240 ng/g cotinine, 600 ng/g nicotine-*N*-glucuronide and OHCOT, 1200 ng/g nicotine, cotinine-*N*-glucuronide, EtS and OHCOT-*O*-glucuronide, and 4800 ng/g EtG

[c]Acidified, methanolic tissue supernatant resulting from filtration, prior to extraction

Table 5 Summary concentration data for alcohol and nicotine markers in fetal liver (*n*=193) and placenta (*n*=48) among positive samples, including minimum, mean, median, and maximum concentrations (ng/g).

		EtG	EtS	*EtG/EtS ratio*	CotG	NicG	OHCotG	OHCOT	COT	NIC	*Individual summations of tobacco markers*
	LOQ (ng/g)	20	5		5	2.5	10	2.5	1	5	
Liver (n=193)	Total positive (n)	18	97	*17*	111	117	51	113	136	105	*137*
	Minimum positive (ng/g)	27.0	5.1	*1.6*	5.2	2.9	10.0	2.5	1.0	5.1	*1.0*
	Mean (ng/g)	303	26.7	*3.9*	192	74.3	25.2	82.0	279	25.1	*592*
	Median (ng/g)	122	13.0	*3.5*	153	54.0	16.0	70.0	126	22.5	*422*
	Maximum (ng/g)	2050	423	*11.1*	745	437	375	313	1332	108	*2776*
Placenta (n=48)	Total positive (n)	16	17	*10*	20	18	10	22	24	22	*25*
	Minimum positive (ng/g)	34.3	6.9	*2.5*	6.4	2.8	15.1	4.4	1.6	5.5	*1.6*
	Mean (ng/g)	253	36.6	*8.7*	80.4	42.2	44.7	97.9	67.6	45.6	*304*
	Median (ng/g)	86.0	17.0	*6.7*	21.6	5.5	28.7	59.5	48.8	22.7	*154*
	Maximum (ng/g)	1168	214	*31.1*	455	467	118	455	221	460	*1621*

Ratio of EtG to EtS is presented for samples with both analytes present. Summations of tobacco markers were also calculated for individual samples.

CotG cotinine-*N*-glucuronide, *NicG* nicotine-*N*-glucuronide, *OHCotG* hydroxycotinine-*N*-glucuronide, *COT* cotinine, *NIC* nicotine

Table 6 Summary of criteria used to stratify evidence of alcohol and tobacco exposure for fetal liver and placenta and number of samples in each category of predicted exposure

	Liver (n = 193)				Placenta (n = 48)				Liver & placenta pairs (n = 47)		
Alcohol	No evidence	Weak evidence	Moderate evidence	Strong evidence	No evidence	Weak evidence	Moderate evidence	Strong evidence	No evidence	Weak evidence	Strong evidence
	EtG & EtS not detected > LOQ	EtG or EtS < median concentration	Absence of EtG, + EtS ≥ median concentration	EtG & EtS detected > LOQ; or EtG ≥ median	EtG & EtS not detected > LOQ	EtG or EtS < median concentration	Absence of EtG, + EtS ≥ median concentration	EtG & EtS detected > LOQ; or EtG ≥ median	Both matrices negative for EtG & EtS	One matrix positive for EtG or EtS	Either matrix positive for EtG & EtS; or both matrices positive for EtG or EtS
	n = 95	n = 36	n = 45	n = 17	n =25	n = 7	n = 4	n = 12	n = 21	n = 15	n = 11
Tobacco	No evidence	Weak evidence	Moderate evidence	Strong evidence	No evidence	Weak evidence	Moderate evidence	Strong evidence	No evidence	Weak evidence	Strong evidence
	No analytes detected > LOQ	1-2 Analytes present < median concentration	≥ 3 Analytes detected but sum < median	All analytes detected; or 5 analytes with sum > median	No analytes detected > LOQ	1-2 Analytes present < median concentration	≥ 3 Analytes detected but sum < median	All analytes detected; or 5 analytes with sum > median	Both matrices negative for all analytes	1-2 Analytes present in liver +/or placenta	≥ 3 Analytes in both matrices; or moderate & strong evidence between the two matrices
	n = 56	n = 23	n = 44	n = 70	n = 23	n = 4	n = 9	n = 12	n = 20	n = 5	n = 22