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DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR THE DETERMINATION OF NICOTINE AND ITS METABOLITES IN PLACENTA AND UMBILICAL CORD

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Abstract

Tobacco exposure during pregnancy is associated with obstetric and fetal complications. We developed and validated an LC-MS/MS method to determine nicotine, cotinine and hydroxycotinine (OH-cotinine) in placenta (PL) and umbilical cord (UC). Specimens were homogenized in water, followed by solid phase extraction. Chromatographic separation was performed using an Atlantis® HILIC Silica column. Detection was accomplished in electrospray in positive mode. Method validation included: linearity (5 to 1000 ng/g), accuracy (86.9 to 105.2% of target concentration in PL, and 89.1 to 105.0% in UC), imprecision (6.8 to 11.8% in PL, and 7.6 to 12.2% in UC), limits of detection (2 ng/g for cotinine and OH-cotinine, and 5 ng/g for nicotine) and quantification (5 ng/g), selectivity (no endogenous or exogenous interferences), matrix effect (-34.1 to -84.5% in PL, %CV=9.1-24.0%; -18.9 to -84.7% in UC, %CV=10.2-23.9%), extraction efficiency (60.7 to 131.5% in PL, and 64.1 to 134.2% in UC), and stability 72 h in the autosampler (<11.5% loss in PL, and <13% loss in UC). The method was applied to 14 PL and UC specimens from tobacco users during pregnancy. Cotinine (6.8-312.2 ng/g in PL; 6.7-342.3 ng/g in UC) was the predominant analyte, followed by OH-cotinine (<LOQ-80.2 ng/g in PL; <LOQ-80.5 ng/g in UC) and nicotine (5.7-63.7 ng/g in PL; 5.1-63.3 ng/g in UC). This method will be applied to more than 150 specimens collected from a wide clinical study to evaluate the usefulness of maternal hair, meconium, placenta and umbilical cord compared to the maternal interview to detect in utero drug exposure.

Keywords: nicotine, placenta, umbilical cord, tobacco, LC-MS/MS

1. Introduction

Active and passive tobacco exposure during pregnancy are associated with short and long term complications, including miscarriage, placental pathologies, decreased birth weight, fetal hypoxia, changes in the fetal heart rate, sudden infant death syndrome (SIDS), learning difficulties, respiratory diseases, increased risk of addictive behavior in adults and increased risk of cancer [1-5].

Surveys about tobacco use in the general population are abundant, and prevalences of tobacco consumption by women of childbearing age can be obtained from these data. The World Health Organization, in its Global Health Observatory (GHO) data, estimates that 19.3% of European women and 6.8% of women worldwide are smokers [6]. In Spain, tobacco is the psychoactive substance with the highest prevalence of daily consumption by females between 15 and 44 years (28.3% of daily consumption during the last month) [7]. However, information on tobacco use among pregnant women is scarce. Currently, the National Survey on Drug Use and Health survey in the USA collects data about prevalence of tobacco consumption by pregnant women. The last National Survey reported that 13.6% of pregnant women aged 15 to 44 in the US smoked tobacco during pregnancy [8].

A direct method to obtain information about drug use during pregnancy is through maternal interviews; nevertheless, this method has low reliability due to poor veracity of maternal answers associated with negative social connotations of drug use during pregnancy [9-12]. A more objective way to identify in-utero drug exposure is the determination of drug biomarkers in biological matrices from the mother, the newborn (blood, urine, meconium), or from the maternal-fetal dyad (placenta, umbilical cord) [13-16].

Meconium is the preferable matrix to detect in utero drug exposure, as it reflects direct fetal exposure during the third trimester of pregnancy [17, 18], providing a window of detection longer than classic matrices such as neonatal blood (hours) or urine (days). Nonetheless, meconium collection may prove challenging, as its expulsion can be delayed up to 5 days after delivery, or it can become unavailable in cases of fetal distress, as discharge may occur before delivery. Maternal hair analysis gives an indirect estimation of fetal drug exposure [17]; however, due to its long window of detection, this matrix could provide information about maternal drug use during the whole

pregnancy, depending on hair's length, thus complementing the information provided by meconium analysis [19].

Alternative matrices to those mentioned before are placenta and umbilical cord. These matrices have several advantages, as they are considered waste products, readily available at the time of birth in abundant amounts, and collection is easy and non-invasive. Although studies conducted to date suggest that placenta and umbilical cord have a larger window of detection than blood or urine [20-25], more studies are needed to assess their usefulness.

To our knowledge, this is the first study to measure nicotine, cotinine and hydroxycotinine (OH-cotinine) levels in both placenta and umbilical cord tissue. Three methods have been published for the determination of tobacco biomarkers in placenta [26-28], and two in umbilical cord [25, 29]. Although these methods have been applied for the analysis of authentic specimens, full method validation data were not reported [27, 28]. Moreover, the reported investigations either included the detection of only nicotine and cotinine [26, 27], or just cotinine [28, 29]; only one article included nicotine, cotinine and OH-cotinine, as well as nornicotine and anabasine [25].

The aim of this work was to develop and validate an LC-MS/MS method for the determination of nicotine, cotinine and OH-cotinine in placenta and umbilical cord. As a proof of concept, the method was applied to authentic placenta and umbilical cord samples from newborns exposed to tobacco during pregnancy.

2. Materials and methods

2.1. Chemicals

Nicotine, cotinine and OH-cotinine standards at 1 mg/mL, and the deuterated internal standards (IStd) nicotine-d_4, cotinine-d_3 and OH-cotinine-d_3 at 0.1 mg/mL in methanol were purchased from Cerilliant™ (Round Rock, TX, USA). Water was purified with a Milli-Q water system (Milli-pore, Le-Mont-sur-Lausanne, Switzerland). Chromasolv® gradient grade methanol and reagent grade dichloromethane were from Sigma-Aldrich (Steinheim, Germany). Chromasolv® LC-MS grade 2-propanol was from Fluka (St. Louis, MO, USA). Reagent grade formic acid 98-100%, reagent grade hydrochloric acid 37% and LC-MS grade ammonium formate were from Scharlau Chemie (Sentmenat, Spain). Ammonium hydroxide 32% and LC-MS grade acetonitrile were from Panreac

(Castellar del Vallés, Spain). Solid phase extraction (SPE) Oasis MCX cartridges (3 cc, 60 mg) were purchased from Waters Corp. (Milford, MA, USA).

2.2. Placenta and umbilical cord samples

Paired placenta, umbilical cord, meconium and maternal hair specimens from more than 800 pregnancies were collected as part of a broad study to evaluate the prevalence of drug use during pregnancy, the usefulness of the different matrices to detect in utero drug exposure, and the possible correlation between drug use and neonatal outcomes. Specimens were collected from pregnant women who delivered at the University Clinical Hospital of Santiago de Compostela and the University Hospital Complex of Vigo, Spain, between 2012 and 2015, and the analyses were performed in our laboratory. The whole placenta and umbilical cord specimens were collected at delivery in polypropylene containers and stored at -20 °C until analysis. The participants were informed about the study both in writing and orally before delivery, and they gave written consent. The subjects were not paid for their participation. The study was approved by the Ethics Committee of the University of Santiago de Compostela, Spain.

For the preparation of calibration curves and quality control samples (QCs), and for the evaluation of method selectivity, blank specimens are needed. Placenta and umbilical cord specimens whose paired maternal hair samples were negative for nicotine and cotinine were initially analyzed to confirm the absence of these analytes.

2.3. Preparation of calibration and QC solutions

For the preparation of the calibration curves, different working solutions in methanol containing all analytes (nicotine, cotinine and OH-cotinine) were generated at 10 µg/mL, 1 and 0.1 µg/mL. Calibration curves were prepared with seven concentration levels (5, 10, 50, 100, 500 and 1000 ng/g) by addition of 50 or 100 µL of the corresponding working solution to placenta or umbilical cord blank samples.

Working solutions at 0.5, 5 and 25 µg/mL in methanol were used for the preparation of low, medium and high concentration QC samples (15, 150 and 750 ng/g, respectively). Thirty µL of the appropriate working solution were added to the blank sample.

Finally, a working solution containing the deuterated internal standards (IStd) (nicotine-d_4, cotinine-d_3 and OH-cotinine-d_3) at 1 µg/mL was prepared by dilution of the original individual ampoules in methanol.

2.4. Sample homogenization

Placenta or umbilical cord was cut into small pieces and 1.00 ± 0.02 g weighed into plastic tubes. Samples were subsequently homogenized in 5 mL purified Mili-Q water with an Ultra-Turrax® disperser at maximum speed until total tissue homogenization. The dispersed samples were transferred into Pyrex® glass tubes, and 50 µL of the IStd at 1 µg/mL and 50 µL of 10% formic acid in water were added. For the calibrators and the QC samples, the homogenized tissue was fortified with the appropriate working solution. The tubes were centrifuged for 15 minutes at 4000 rpm. All the supernatant was collected and subjected to SPE.

2.5. Solid phase extraction procedure

SPE was performed with Oasis MCX cartridges (3 cc, 60 mg). Cartridges were conditioned with 2 mL methanol and 2 mL water. The sample was loaded in two steps due to the 3 cc volume limitation of the cartridges. Cartridges were subsequently washed with 2 mL 0.1% formic acid in water (v:v). After drying the cartridges for 20 min, analytes were eluted with 3 mL dichloromethane:2-propanol:ammonium hydroxide (23.75:71.25:5, v:v:v). Samples were then acidificated by addition of 100 µL 1% hydrochloric acid in methanol (v:v) to prevent evaporation of analytes. Eluates were evaporated using a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA) and reconstituted in 200 µL acetonitrile with 0.1% formic acid:methanol (3:1, v:v). After centrifugation for 10 minutes at 14000 rpm, extracts were transferred into injection vials, and 20 µL were injected for LC-MS/MS analysis.

2.6. LC-MS/MS

An Alliance 2795 Separation Module with an Alliance series column heater/cooler (Waters Corp.) was employed for the chromatographic separation using an Atlantis® HILIC Silica (2.1 mm x 100 mm, 3 µm) column (Waters Corp.), maintained at 30 ºC. Formic acid (0.1%) and 2 mM ammonium formate in water (A) and 0.1% formic acid in acetonitrile (B) were used as mobile phase at a flow rate of 0.3 mL/min. The chromatographic gradient was programmed as follows: 5% A linearly increased to 60% until min 10; return to initial conditions at min 10.5, and equilibrate until min 15. The autosampler temperature was maintained at 6 ºC.

The mass spectrometer employed was a Quattro Micro™ API ESCI triple quadrupole (Waters Corp.). The instrument was operated in electrospray in positive mode (ESI+) with the following optimized settings: capillary voltage 3.0 kV; source block and

desolvation gas (nitrogen) temperature 130 ºC and 300 ºC, respectively; desolvation and cone gas (nitrogen) flow rate 500 L/h and 50 L/h, respectively. Data were recorded on multiple reaction monitoring (MRM) mode. A 10 µg/mL post-column infusion of each individual analyte at 100 µL/min connected with a "T" valve to the chromatographic effluent (0.1% formic acid in water:ACN, 50:50, v:v) was employed to select MRM transitions, cone voltages and collision energies for the analytes of interest and IStds. MassLynx 4.0 software was employed to control data acquisition and QuanLynx 4.1 for data processing (Waters Corp.).

2.7. Method validation

Method validation was performed according to the Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology [30] and the European Medicines Agency (EMA) guideline on bioanalytical method validation [31]. The following paramenters were evaluated for method validation: linearity, accuracy, imprecision, limit of detection (LOD) and limit of quantification (LOQ), selectivity, matrix effect, extraction efficiency, process efficiency and stability in the autosampler for 72h. All parameters were studied in placenta and in umbilical cord.

Linearity was assessed by the evaluation of calibration curves with 7 calibration levels analyzed on four different days. Concentration ranges were from 5 to 1000 ng/g for all analytes. The straight-line fit was performed by linear regression, applying a 1/x-weighting factor. Linearity was acceptable if coefficient of determination (r^2) was $\geqslant$0.99, and calibrators' residuals ±15%, except for the LOQ, for which residuals ±20% were accepted.

Accuracy and imprecision were evaluated at low, medium and high QC concentrations (15, 150 and 750 ng/g, respectively). These parameters were assessed by the analysis of 5 replicates for each QC analyzed on 4 different days (n=20). Accuracy was required to be within 85-115% of the nominal concentration (80-120% for the LOQ). Intra-assay, inter-assay and total imprecision were determined by calculating the coefficient of variation (%CV) following Krouwer and Rabinowitz' recommendations [32], and using SPSS v. 20.0 statistical software. Requirement of %CV was to be less than 15% (20% for the LOQ).

The limit of detection (LOD) was defined as the lowest concentration at which the two MRM transitions monitored for each analyte can be identified with a S/N (signal-to-noise ratio) $\geqslant$3, an appropriate ion ratio, and within ±0.2 min of the mean calibrators retention time.

The limit of quantification (LOQ) was defined as the lowest concentration that could be quantified with an imprecision (% CV) $\leq$20% and accuracy between 80 and 120% of the theoretical value. The LOQ was evaluated by the analysis of 5 replicates of blank placenta samples and 5 replicates of blank umbilical cord samples from different individuals fortified at the lowest concentration of the calibration curve.

Selectivity of the method was evaluated for both exogenous and endogenous interferences. To evaluate the effect of endogenous interferences, blank placenta and umbilical cord samples from 10 different individuals were fortified with the IStds and analyzed. To evaluate the presence of exogenous interferences, blank placenta and umbilical cord samples were fortified with common drugs of abuse and medicines (morphine, codeine, 6-acetylmorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, amphetamine, methamphetamine, 3,4-methylendioxyamphetamine, 3,4-methylendioxymethamphetamine, 3,4-methylendioxyethylamphetamine, cocaine, benzoylecgonine, ecgonine methylester, cocaethylene, lysergic acid diethylamide, ketamine, norketamine, gammahydroxybutyric acid, fentanyl, amitriptyline, paroxetine, zolpidem, zopiclone, ibuprofen, omeprazole, paracetamol, diclofenac, naproxen, alprazolam, temazepam, lormetazepam, lorazepam, clonazepam, diazepam, nordiazepam, flunitrazepam, 7-aminoflunitrazepam, oxazepam, triazolam, nitrazepam, bromazepam) at 1000 ng/g.

Matrix effect, extraction efficiency and process efficiency were evaluated in placenta and in umbilical cord at low and high QC concentrations (15 and 750 ng/g, respectively), following recommendations published by Matuszewski et al. [33]. Evaluation of matrix effect was performed by comparing average analyte peak area in blank samples from 10 different individuals fortified with the analytes after extraction, with average peak area of the analytes prepared at the same concentration in acetonitrile with 0.1% formic acid:methanol (3:1, v:v) (n=5). Extraction efficiency was evaluated by comparing average analyte peak area in blank samples fortified with the analytes before extraction (n=5) with average peak area obtained in blank samples fortified after extraction (n=10). Process efficiency was calculated by comparing average analyte peak

area in blank samples fortified with the analytes before extraction (n=5) with average peak area of the analytes prepared at the same concentration in acetonitrile with 0.1% formic acid:methanol (3:1, v:v) (n=5).

Autosampler stability of the compounds was evaluated at low, medium and high QC concentrations by comparing concentrations obtained after the injection of freshly prepared QC samples (n=5) and after reinjection 72 hours later. The stability was considered acceptable if the reinjected QCs were quantified within ±15% compared to freshly prepared QCs.

2.8. Application to authentic samples

To confirm method applicability, 14 authentic umbilical cord and placenta samples from pregnant tobacco users were analyzed. Tobacco use was confirmed by the analysis of the paired maternal hair specimen using a previously published method which was further expanded to include nicotine and cotinine determination [34]. Hair specimens were divided into 3 segments corresponding with the 3 trimesters of pregnancy. Segment 1, corresponding to the last trimester of pregnancy, was from 0 (root) to 2 cm; segment 2, corresponding to the 2nd trimester, from 3 to 6 cm; and segment 3, corresponding to the 1st trimester, was from 6 to 9 cm. However, if the amount of hair specimen was not enough for segmentation only one segment up to 8 cm long, corresponding to the whole pregnancy, was analyzed.

To ensure that the analysis of a single intermediate location would be accurately representative of placental and umbilical cord disposition, we initially assessed whether the analytes of interest were homogeneously distributed throughout the tissues. For this purpose, analytes concentrations in 9 placenta samples at 4 different locations (1, 4, 6, and 10 cm from the umbilical cord) and in the paired umbilical cord at two locations (start and end of the tissue) were evaluated.

3. Results

3.1. Method development

The present analytical methods allowed the determination of nicotine, cotinine and OH-cotinine in 1 g placenta or umbilical cord. Both methods employed the same analytical procedure, including chromatographic separation, and sample homogenization and extraction, and they were completely validated in each matrix. Chromatographic elution

of all the analytes was achieved in 7.5 min, with a total chromatographic run time of 15 min.

The most abundant MRM transition was used for quantification. A second transition was monitored for qualitative purposes to fulfill the European Commission Decision 2002/657/EC identification criteria using mass spectrometric techniques [35].

Table 1 shows quantification and qualification transitions, cone voltage, collision energy and retention time for each analyte.

3.2. Method validation

Selectivity of the method was verified as no quantifiable endogenous or exogenous interferences were detected at the specific retention time for each analyte in 10 different blank placenta and umbilical cord specimens (Figures 1a and 2a), or in the blank samples fortified with common drugs of abuse and medicines.

LOD was 5 ng/g for nicotine, and 2 ng/g for the metabolites, and the LOQ was 5 ng/g for all the analytes in both matrices. Figures 1b and 2b correspond to blank placenta and umbilical cord samples fortified at the LOQ.

Linearity of the compound-to-IStd ratio versus the theoretical concentration was verified through 4 calibration curves analyzed in 4 different days with 1/x weighting factor. For all the analytes the curves were fitted to a linear regression model over the concentration range of 5 to 1000 ng/g, obtaining a mean $r^2 \geq 0.995$ for all the analytes. Residuals were ±20 % at the LOQ and ±15 % for the rest of the calibrators. Table 2 shows linearity range and calibration parameters results for nicotine, cotinine and OH-cotinine in placenta and in umbilical cord.

Results for accuracy and imprecision in placenta and umbilical cord are shown in Table 3. Accuracy was satisfactory for all the analytes in both matrices (86.9-105.2% of the target concentration in placenta, and 89.1-105.0% in umbilical cord). Intra-assay, inter-assay and total imprecision were <6.8%, <10.3% and <11.8%, respectively, in placenta, and <7.6%, <9.6% and <12.2% in umbilical cord.

Matrix effect, extraction efficiency and process efficiency results are shown in Table 4. All analytes showed ion suppression at low and high QC concentrations in both matrices. Matrix effect ranged from -34.1 to -84.5% (%CV=9.1-24.0%) in placenta, and from -18.9 to -84.7% (%CV=10.2-23.9%) in umbilical cord. In all cases, behavior of the deuterated internal standards was similar to that observed for the non-labeled analyte,

compensating matrix effect results. Extraction efficiency ranged from 60.7 to 99.5% in placenta, and from 64.1 to 103.9% in umbilical cord, except for nicotine-d4, for which values up to 131.5% and 134.2% were observed in placenta and in umbilical cord, respectively. These high extraction efficiency values were probably due to the higher variability observed in the different specimens used to evaluate this parameter compared to those employed to calculate the matrix effect for nicotine-d4. %CV for the 5 replicates ranged from 12.7 to 29.9% in placenta, and 10.5 to 25.3% in umbilical cord. Overall process efficiency ranged from 13.2 to 59.6% and from 14.7 to 57.0% in placenta and umbilical cord, respectively.

Results from the stability study in the autosampler showed that all the analytes were stable in these conditions for 72 h, with <13% loss when compared to fresh QC samples in all cases.

3.3. Application to authentic samples

Table 5 shows results observed in the experiment to evaluate whether the analytes were homogeneously distributed in 9 pairs of placenta and umbilical cord specimens (samples A to I). %CV for nicotine, cotinine and OH-cotinine concentrations observed at the 4 different placenta locations was <15%, except for OH-cotinine in placenta E (%CV= 22.3%), and nicotine in placenta F (%CV=20.9%) . In umbilical cord, %CV was <15% in all cases, except for nicotine in umbilical cord D (%CV= 28.0%).

Due to the homogeneous distributions, a single intermediate location was analyzed for all the other placenta and umbilical cord specimens. Eight out of 14 placenta and umbilical cord specimens were positive for the 3 analytes (Table 5). Similar concentrations were observed in placenta and in umbilical cord. Nicotine, cotinine and OH-cotinine concentrations in placenta ranged from 5.7 to 63.7 ng/g, 6.8 to 312.2 ng/g and <LOQ to 80.2 ng/g, respectively; and concentrations in umbilical cord ranged from 5.1 to 63.3 ng/g, 6.7 to 342.3 ng/g and <LOQ to 80.5 ng/g, respectively. Hair concentrations were decreasing throughout the pregnancy for participants for whom a segmental analysis could be performed, with the lowest concentrations observed in the third trimester, except for participant I (data not shown). For participants B, L, M and N only one segment of 8 cm length could be analyzed. Eight out of 14 hair specimens were positive for both nicotine and cotinine; however, as opposed to placenta and umbilical cord, the parent drug was the predominant analyte in hair, except for

participant A. Hair concentrations were much higher than those found in placenta and in umbilical cord, ranging from 265.9 to 15428.3 ng/g for nicotine, and from 72.7 to 668.5 ng/g for cotinine; however, hair concentrations were not correlated to those observed in placenta and in umbilical cord (Table 5). Figure 3 shows the chromatogram of the 2 MRM transitions identified for each analyte after the analysis of the placenta and the umbilical cord of the real case L.

4. Discussion

The present analytical method allows for the simultaneous quantification of nicotine and its main metabolites cotinine and OH-cotinine in placenta and umbilical cord using LC-MS/MS. For this purpose, samples were homogenized with water and extracted with solid phase extraction columns based on mixed mode reversed-phase and cation exchange mechanisms. Chromatographic separation was performed in 7.5 min using a HILIC column, and a LOQ of 5 ng/g was achieved with these conditions. Although a concentration of 2 ng/g in placenta or umbilical cord fulfilled the criteria for the LOD for the 3 analytes, we increased this value to 5 ng/g for nicotine as a minimal non-quantifiable contamination for this compound was observed in the blank samples.

For placenta and umbilical cord homogenization we tested several solutions, including water, 10% formic acid and methanol. Best results were obtained with water, as samples homogenized with formic acid prompted cartridge clogging, and those mixed with methanol were more difficult to blend. Samples were subsequently extracted by SPE. Different cartridges were evaluated for sample extraction, including OASIS MCX 3 cc 60 mg, OASIS HLB 3 cc 60 mg (Waters Corp., Mildford, MA, USA) and Strata-X-C 3 cc 60 mg (Phenomenex, Torrance, CA, USA), with the best results in terms of sensitivity observed for mixed mode reversed phase-cation exchange cartridges. OASIS MCX were finally selected, as cartridge clogging was usually observed when placenta or umbilical cord samples were extracted with Strata-X-C. To optimize analytes elution we assessed several combinations of NH_4OH and methanol, and dichloromethane, 2-propanol and NH_4OH. Best results were obtained with dichloromethane:2-propanol:NH_4OH (23.75:71.25:5, v:v:v).

For chromatographic separation, best results were observed using a HILIC analytical column. We also assayed several reversed-phase analytical columns; however, due to the polarity of the analytes, retention was very poor.

Some analytical methods have been reported for the determination of nicotine and/or its metabolites in placenta and/or umbilical cord by GC or LC-MS [25-28]. Marin et al. [25] developed a method for the determination of nicotine and several metabolites including cotinine, OH-cotinine, nornicotine and anabasine in meconium and umbilical cord by LC-MS/MS. The samples (0.25 g of meconium or 1.5-2 g of umbilical cord) were homogenized with methanol, and afterwards extracted with mixed mode reversed-phase cation exchange columns, although they employed Trace B instead OASIS MCX columns. Chromatographic separation was performed using a HILIC analytical column. Limits of quantification in umbilical cord were lower than those described in the present manuscript (0.5 ng/g for nicotine and OH-cotinine, and 0.25 ng/g for cotinine, nornicotine and anabasine). Concentrations found in 14 umbilical cord specimens from mothers who admitted smoking during the last trimester were higher than our LOQ for cotinine and OH-cotinine (>5 and >17 ng/g, respectively); however, nicotine concentrations in these cases were between 0.9 and 11.7 ng/g. Unfortunately, method validation was not described and, therefore, selectivity, and precision and accuracy of the quantitative results could not be assessed. Joya et al. [26] published a method for the determination of drugs of abuse including amphetamine and derivatives, opioids, cocaine and cannabis, nicotine and cotinine in placenta from women that voluntarily interrupted their pregnancy during the first trimester. Samples were homogenized with $HClO_4$ 0.1%, and subsequently extracted with Strata-X-C cation exchange cartridges. The authors used the same amount of placenta reported in the present study (1 g), and achieved similar limits of quantification (13.9 ng/g for nicotine and 2.1 ng/g for cotinine) and limits of detection (4.6 ng/g for nicotine and 0.7 ng/g for cotinine). The authors describe the results of some validation parameters (linearity, analytical recovery, LOQ, LOD, precision and accuracy, and stability). Recently, Mohammadi et al. [27] published a method for the determination of nicotine and cotinine, and several polycyclic aromatic hydrocarbons (PAHs) and tobacco-specific nitrosamines in placenta by UPLC-QTOF-MS. After placenta (5 g) protein precipitation with trichloroacetic acid, samples were blended and also extracted with cation exchange cartridges. A reversed-phase analytical column (ACQUITY UPLC BEH C18) was employed for the chromatographic separation. High LOQs were obtained, with values of 88 and 26 ng/g for nicotine and cotinine, respectively. LODs were also high (27 ng/g for nicotine and 8 ng/g for cotinine). Unfortunately, method validation included only extraction recovery and matrix effect. Finally, Mamsen et al. [28] reported an LC-MS/MS method for the

410 determination of cotinine and perfluorinated compounds (PFASs) in placenta, fetal
411 tissues and maternal plasma, as maternal cigarette smoking may affect PFASs
412 concentrations. However, calibration curves in plasma were used for the quantification
413 of the analytes found in all type of samples, and no method validation was reported in
414 any of the matrices.

415 The present method was completely validated in placenta and in umbilical cord. Our
416 method fulfilled the acceptance values for all the studied validation parameters,
417 including selectivity, linearity, precision, accuracy, extraction efficiency, matrix effect
418 and autosampler stability. In addition, real placenta and umbilical cord specimens were
419 analyzed to prove the method applicability. The homogeneous distribution in placenta
420 and in umbilical cord have been previously described for several drugs, including
421 cocaine, opiates and methadone and metabolites [36, 37]. Concheiro et al. found a
422 homogeneous distribution in these tissues for buprenorphine (BUP) metabolites BUP
423 glucuronide, norBUP and norBUP glucuronide in placenta [38] and in umbilical cord
424 [39]. However, BUP was not detected in some locations in 2 out of 5 placenta
425 specimens; this was probably due to the low concentrations of BUP in the specimens (1-
426 2.4 ng/mL) that were close to the method LOQ (1 ng/mL). BUP was never detected in
427 umbilical cord. In the present manuscript, we confirmed that nicotine and metabolites
428 concentrations in different tissue locations are homogeneous and, therefore, one single
429 intermediate location of the real specimens was analyzed. LOD and LOQ for the three
430 analytes in placenta and umbilical cord were sufficient for identification of in utero fetal
431 exposure, as at least 2 nicotine biomarkers were detected in the 14 authentic cases
432 collected at delivery from tobacco user mothers. Similar concentrations were found in
433 placenta and umbilical cord. Cotinine (6.8-312.2 ng/g in placenta; 6.7-342.3 ng/g in
434 umbilical cord) was the predominant analyte, followed by OH-cotinine (<LOQ-80.2
435 ng/g in placenta; <LOQ-80.5 ng/g in umbilical cord) and nicotine (5.7-63.7 ng/g in
436 placenta; 5.1-63.3 ng/g in umbilical cord). Concentrations found in paired hair
437 specimens were much higher than those observed in placenta and in umbilical cord;
438 moreover, as expected, the parent drug was usually the predominant analyte in hair. For
439 4 participants only one segment of 8 cm long corresponding to drug intake for the whole
440 pregnancy could be analyzed. Therefore, hair concentrations corresponding to the third
441 trimester of pregnancy were only available for 10 participants. In these cases, nicotine
442 and cotinine concentrations in hair were not associated to those found in placenta and

umbilical cord, as the highest concentrations in hair were observed for participant I followed by participants K>F>J>C>G>E>D>A>H, while the highest concentrations in placenta and in umbilical cord were found for participant C followed by participants F>D>G>A>K>E>J>I>H.

Joya et al. [26] detected 21 positive placenta specimens (32.8% of the analyzed specimens) by the identification of cotinine (24.7-189.6 ng/g, median= 80.4 ng/g), and nicotine in some cases (32.5-119.5 ng/g, median=61.2 ng/g). The reason for the higher nicotine concentrations compared to those found in our specimens could be the origin of the samples, which were donated by women that voluntarily interrupted their pregnancy during the first trimester of pregnancy, and as previous studies showed, this is associated with higher rates of maternal smoking [8, 40, 41]. Marin et al. [25] compared nicotine and metabolite profile in 14 paired meconium and umbilical cord specimens from women that admitted tobacco consumption during their pregnancies. Cotinine (14.2-157.7 ng/g) and OH-cotinine (26.6-195.2 ng/g) were also the predominant analytes in umbilical cord, although nicotine and norcotinine were also detected in 12 cases (0.9-11.7 ng/g and 0.3-1.3 ng/g, respectively). The same analytes were detected in meconium, although at higher concentrations and with a different metabolite profile, as in this matrix the predominant analytes were usually nicotine (20.8-590.1 ng/g) and OH-cotinine (34.9-354.2 ng/g). Mohammadi et al. [27] found cotinine (17.2-61.8 ng/g) and nicotine (27-88 ng/g) in 8 umbilical cord specimens from smoking mothers; and, as expected, none of the analytes were identified in the 18 specimens from mothers that did not smoke during the pregnancy. Finally, Mansem et al. [28] reported cotinine concentrations of 4-375 ng/g in 14 placenta specimens from smoking mothers.

The present LC-MS/MS method was developed for the identification of newborns exposed to tobacco during the pregnancy by the analysis of placenta and umbilical cord. Its main limitation is that the sensitivity for nicotine could compromise its detection in some cases; however, in these cases, identification of in utero exposure would be guaranteed by the detection of its metabolites cotinine and/or OH-cotinine. The method will be applied to more than 150 placenta and umbilical cord specimens collected at delivery from mothers whose hair tested positive for nicotine and/or cotinine. This will increase our knowledge on nicotine and metabolites disposition in placenta and umbilical cord, and the usefulness of these alternative matrices for identification of in utero tobacco exposure.

5. Conclusion

To our knowledge, this is the first analytical method for the simultaneous determination of nicotine, cotinine and OH-cotinine in both placenta and umbilical cord. The methods were successfully validated, achieving satisfactory results for all the studied parameters. Analysis of authentic placenta and umbilical cord specimens from pregnant women whose hair tested positive to tobacco confirmed the applicability of these analytical methods, showing cotinine as the predominant analyte, with similar concentrations observed in both matrices. The present methods will be applied to more than 150 paired placenta and umbilical cord specimens to evaluate the usefulness of these alternative matrices to detect of in utero drug exposure, and to assess a possible correlation between detected concentrations and neonatal outcomes.

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Table 1. MRM transitions, cone voltage (CV), collision energy (CE), retention time (Rt) for each analyte.

Analyte	MRM transition	CV (V)	CE (eV)	Rt (min)
Nicotine	163.1 > 132.2 163.1 > 116.7	30	18 26	7.2
Nicotine-d4	167.2 > 120.7	30	28	7.3
Cotinine	177.1 > 79.5 177.1 > 97.6	35	22 20	2.8
Cotinine-d3	180.2 > 79.5	35	24	2.9
OH-Cotinine	193.2 > 133.8 193.2 > 79.5	30	18 24	2.6
OH-Cotinine-d3	196.1 > 79.5	35	24	2.7
Underlined transitions were used for quantification. OH-Cotinine: hydroxycotinine				

Table 2. Linearity range and calibration parameters for nicotine, cotinine and hydroxycotinine (OH-cotinine) in placenta and umbilical cord.

		Placenta			Umbilical cord		
Analyte	Linearity range (ng/g)	Intercept ± SD (n = 4)	Slope ± SD (n = 4)	r^2 ± SD (n=4)	Intercept ± SD (n = 4)	Slope ± SD (n = 4)	r^2 ± SD (n=4)
Nicotine	5-1000	1.52 ± 2.77	1.23 ± 0.53	0.9991 ± 0.0008	1.82 ± 2.11	0.84 ± 0.23	0.9984 ± 0.0010
Cotinine	5-1000	2.85 ± 5.76	1.22 ± 0.22	0.9978 ± 0.0014	2.94 ± 3.59	1.18 ± 0.27	0.9992 ± 0.0006
OH-Cotinine	5-1000	4.86 ± 3.57	2.48 ± 1.12	0.9969 ± 0.0018	7.37 ± 4.78	2.36 ± 0.48	0.9992 ± 0.0004

Table 3. Results for imprecision and accuracy in placenta and umbilical cord at low (15 ng/g), medium (150 ng/g) and high (750 ng/g) QC concentrations.

Analyte	Matrix	Intra-assay imprecision (n =20; %CV)			Inter-assay imprecision (n =20; %CV)			Total imprecision (n = 20; %CV)			Accuracy (n =20; % target concentration)		
		15 ng/g	150 ng/g	750 ng/g	15 ng/g	150 ng/g	750 ng/g	15 ng/g	150 ng/g	750 ng/g	15 ng/g	150 ng/g	750 ng/g
Nicotine	PL	6.8	2.5	3.0	8.6	9.7	4.3	10.9	10.0	5.2	105.2	92.0	86.9
	UC	3.7	4.4	4.6	9.3	2.8	2.0	10.0	5.2	5.0	97.8	93.0	91.6
Cotinine	PL	5.3	3.9	3.2	2.5	3.4	4.3	5.9	5.2	5.4	102.6	104.2	93.1
	UC	4.1	3.2	4.2	6.1	4.7	7.2	7.4	5.7	8.3	105.0	94.0	96.3
OH-Cotinine	PL	5.9	4.0	5.5	10.3	7.5	7.5	11.8	8.5	9.3	93.5	89.1	90.2
	UC	7.6	4.0	2.2	9.6	4.4	9.2	12.2	6.0	9.4	98.5	89.1	91.3
OH-Cotinine: hydroxycotinine; PL: placenta; UC: umbilical cord													

673 Table 4. Matrix effect, extraction efficiency and process efficiency results in placenta
674 and umbilical cord at low (15 ng/g) and high (750 ng/g) QC concentrations.

Matrix	Analyte	Matrix effect (%) (%CV) (n=10)		Extraction efficiency (%) (%CV) (n=6)		Process efficiency (%) (n=6)	
		15 ng/g	750 ng/g	15 ng/g	750 ng/g	15 ng/g	750 ng/g
Placenta	Nicotine	-84.5 (19.4)	-71.5 (9.1)	87.5 (25.6)	82.6 (15.7)	13.2	24.5
	Nicotine-d4	-81.6 (19.5)	-68.9 (14.4)	131.5 (23.2)	114.1 (18.1)	23.5	36.8
	Cotinine	-45.8 (20.2)	-36.8 (11.7)	88.7 (24.2)	84.2 (12.7)	49.1	53.4
	Cotinine-d3	-45.1 (20.6)	-39.7 (14.7)	90.5 (22.1)	93.1 (15.4)	50.6	54.0
	OH-Cotinine	-50.7 (23.8)	-34.1 (12.6)	89.9 (29.9)	60.7 (24.3)	44.6	40.1
	OH-Cotinine-d3	-51.9 (24.0)	-36.4 (16.4)	99.5 (28.1)	96.7 (26.9)	48.2	59.6
Umbilical cord	Nicotine	-84.7 (17.3)	-72.0 (10.2)	96.3 (22.5)	87.4 (15.7)	14.7	24.4
	Nicotine-d4	-82.6 (17.2)	-68.9 (15.5)	134.2 (23.9)	118.0 (19.1)	23.4	36.7
	Cotinine	-44.5 (19.1)	-36.8 (13.1)	93.3 (23.3)	87.7 (10.5)	51.7	55.4
	Cotinine-d3	-21.1 (19.3)	-18.9 (16.6)	96.7 (21.9)	91.8 (13.5)	53.6	54.0
	OH-Cotinine	-50.7 (23.9)	-37.2 (14.0)	92.5 (25.3)	64.1 (13.8)	45.6	40.3
	OH-Cotinine-d3	-52.9 (22.9)	-40.2 (16.8)	103.9 (24.2)	95.4 (24.1)	48.9	57.0
OH-Cotinine: hydroxycotinine							

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Table 5. Average analyte concentrations (ng/g) and %CV found in 4 different placenta locations and 2 different umbilical cord locations from participants A to I. Analyte concentrations (ng/g) in placenta and umbilical cord specimens from participants J to N. Paired hair concentrations found for nicotine and cotinine in the third trimester are expressed in ng/g.

Case	Placenta			Umbilical cord			Hair	
	Nicotine	Cotinine	OH-Cotinine	Nicotine	Cotinine	OH-Cotinine	Nicotine	Cotinine
A	<LOD	46.2 (3.1%)	7.0 (6.3%)	<LOD	52.8 (2.9%)	5.8 (3.6%)	<LOD	72.7
B	63.7 (3.2%)	136.2 (2.7%)	58.1 (2.1%)	63.3 (6.4%)	152.4 (1%)	57.8 (4.5%)	*10297.7	*323.2
C	20.2 (11.5%)	312.2 (5.2%)	80.2 (4.7%)	20.9 (12.9%)	342.3 (2.3%)	80.5 (2%)	1447.8	433.8
D	9.7 (7.2%)	50.7 (1.3%)	11.3 (7.1%)	13.8 (28.0%)	59.9 (1.2%)	13.6 (10.9%)	265.9	<LOD
E	<LOD	38.6 (3.5%)	12.6 (22.3%)	<LOD	45.9 (6.4%)	16.4 (1.2%)	541.5	109.4
F	9.6 (20.9%)	203.7 (10.7%)	59.7 (10.2%)	5.1 (1.4%)	108.1 (1.1%)	35.0 (0.2%)	3403.3	339.4
G	<LOD	45.1 (11.1%)	11.9 (14.4%)	8.2 (2.6%)	59.0 (5.1%)	17.3 (8.2%)	1167.2	416.3
H	<LOD	<LOQ	<LOQ	<LOD	6.7 (14.7%)	<LOQ	<LOD	<LOD
I	<LOD	7.9 (6.8%)	<LOQ	9.3 (13.0%)	8.7 (0.8%)	5.6 (0.0%)	15428.3	668.5
J	6.7	23.1	19.3	8.8	18.5	17.5	2705.4	168.4
K	8.5	39.7	18.8	12.8	50.2	38.6	10313.1	<LOD
L	7.6	17.6	16.6	9.6	20.6	28.8	*1733.5	*<LOD
M	5.7	7.1	8.3	6.4	10.6	12.9	*1770.5	*<LOD
N	<LOD	6.8	<LOD	7.8	9.7	6.7	*1845.1	*112.7
OH-cotinine: hydroxycotinine; *Only 1 segment representing the whole pregnancy was analyzed.								

Figure captions

Figure 1. Chromatograms of the MRM transitions for nicotine, cotinine and hydroxycotinine (OH-cotinine) in a blank placenta sample (1a), and in placenta fortified at the LOQ (5 ng/g) (1b).

Figure 2. Chromatograms of the MRM transitions for nicotine, cotinine and hydroxycotinine (OH-cotinine) in a blank umbilical cord sample (2a), and in umbilical cord fortified at the LOQ (5 ng/g) (2b).

Figure 3. Chromatograms of the MRM transitions for nicotine, cotinine and hydroxycotinine (OH-cotinine) in the placenta (3a) and umbilical cord (3b) from a positive real specimen (Case L). Nicotine, cotinine and OH-cotinine concentrations were 7.6, 17.6 and 16.6 ng/g, respectively, in placenta; and 9.6, 20.6 and 28.8 ng/g in umbilical cord.