Review
Special Focus Issue: Special Populations

Bioanalysis

Bioanalysis during pregnancy: recent advances and novel sampling strategies

Consumption of drugs of abuse, tobacco and alcohol throughout pregnancy is a serious public health problem and results in an important economic cost to the health system. Drug and/or metabolites determination in biological matrices from mother and newborn is an objective measure of *in utero* drug exposure. We reviewed methods published for the determination of *in utero* drug exposure from 2007 to 2014, with special focus on meconium, placenta, umbilical cord and newborn hair. Accurate bioanalytical procedures are essential to obtain high-quality data to perform interventions and to establish correlations between analytical measures and clinical outcomes. We included a brief overview of clinical implications of *in utero* drug exposure to better understand the importance of this serious health issue.

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The consumption of drugs of abuse during pregnancy constitutes a major public health concern. Prenatal exposure to alcohol, tobacco and drugs of abuse has been associated with deleterious short- and long-term effects in exposed children [1,2]. Seventy-five percent of the exposed newborns develop these deleterious effects, compared with 27% of the nonexposed [3]. In addition, 17% of the exposed children are preterm compared with 6% of the nonexposed [4]. The kind of harm depends on the trimester of pregnancy when the drug exposure takes place. Heavy drug use during the first trimester often results in spontaneous abortions [5]. Also, in this period, the fetus is most susceptible to teratogens [6]. In the second and third trimesters, the main harmful effects are related to fetal growth and maturation. Preterm low birth weight newborns have a higher morbidity and mortality than do older, heavier fetuses [1]. This is the case because preterm newborn organs, such as liver and kidneys, are immature and, therefore, drug elimination is slower than in older newborns. Also because of the low weight at birth, the distribution volume is lower than in heavier newborns, and drug concentrations in plasma are expected to be higher.

In the USA, 15.9% of pregnant women self-reported tobacco use in the last month, while 8.5% reported drinking alcohol and 5.9% reported using illicit drugs. Illicit drugs include marijuana, cocaine, heroin, methamphetamine and other stimulants and nonmedical use of prescription medicines [7]. Unfortunately, this kind of data are scarce in other countries. In Spain, 34% women between 15 and 64 years old consumed tobacco during the last month, 52.2% alcohol and 5.3% illicit drugs (marijuana, cocaine, amphetamines, hallucinogens and heroin) [8].

Personal interview is the method employed in many clinical settings to detect drug consumption during pregnancy. Although this approach is easy and affordable, it is often not reliable. Mothers usually under-report usage because of their fear of the legal and social consequences that would follow if they confessed their addiction problems [9,10], or because they are not aware of the risk. The determination of drugs and/or metabolites in biological specimens from the mother (urine, blood/plasma, oral fluid, sweat and hair), from the newborn (urine, meconium and hair) and from the maternal-fetal unit (placenta and umbilical cord) offers an objective measure of this exposure. Previously published reviews discussed

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Key term

Neonatal abstinence syndrome: Neonatal withdrawal after intrauterine exposure to illegal or prescription drugs. It occurs with the abrupt cessation of the drug exposure at birth.

the main advantages and disadvantages for some of these matrices [11–14]. These reviews summarized the methods published until 2007, and important matrices such as placenta and umbilical cord were not included.

In the present review, we revised the most recent publications (2007–2014) in bioanalytical methods for the determination of exposure to alcohol, tobacco and drugs of abuse during pregnancy in different biological matrices. We also included an overview of the clinical implications of the drug exposure in this high-risk population to understand the importance of this serious health problem. Articles related to analytical methods to detect *in utero* drug exposure were searched in PubMed and Web of Science for January 2007 to April 2014. The articles were manually checked on content and then further checked for cross-references. The articles were limited to the English language.

Effects of prenatal exposure to drugs of abuse, tobacco & alcohol

Prenatal drug use has been associated with potentially deleterious and even long-term effects on exposed children. However, estimating the full extent of the consequences of maternal drug abuse is difficult as multiple genetic, epigenetic and environmental factors make it difficult to determine the direct impact of prenatal drug exposure on the child. Besides, the fetus can be exposed to the effect of one or multiple drugs at the same time. The effects of prenatal drugs on the developing fetus are complex and modulated by the timing (trimester of pregnancy), amount, duration and route of drug exposure. Mother and child blood circulation are connected via umbilical cord and placenta and drugs in mother's blood will reach the fetus in an increased concentration due to his little distribution volume. The deleterious effects of some drugs (e.g., nicotine, cocaine) in the fetus may derive from damage of the placental structure that nourishes and oxygenates him. Fetal growth disruption appears and derives in low birth weight. Other mechanism of harm is a direct teratogenic effect expressed as anomalies in fetus organs after consumption during critical periods of differentiation and growth (first trimester). Fetal Alcohol Spectrum Disorder (FASD) is linked to alcohol exposition and characterized by a peculiar phenotype, congenital cardiopathy, microcephaly and neurocognitive impairment as a consequence of an anomalous development of the brain [15]. Antidepressant and antiepileptic drugs may also lead to congenital anomalies, and smoking has been associated with oral cleft. Other drugs (cocaine, cannabioids, methamphetamine) may interfere with brain microstructure development or alter neurotransmission pathways and lead to cognitive and behavioral deficits later on [16].

Pregnancy outcome

Drug use in pregnancy appears to increase the risk of obstetrical complications like miscarriage, preterm delivery, deep vein thrombosis, pregnancy-induced hypertension and antepartum hemorrhage (placental abruption). Most of these are related to placental damage derived from vasoconstrictor effects of some drugs (e.g., cocaine, methamphetamines, nicotine) or from direct trophoblastic citotoxicity (e.g., ethanol) [17]. However, mother nutritional status, chronic virus C hepatitis infection, prenatal care, socioeconomic conditions and many other variables related to drug consumption may also interfere with the normal course of pregnancy.

Short-term effects in the neonate (neonatal abstinence syndrome)

After prolonged intra-utero exposition, withdrawal symptoms may appear at birth when the passage of drugs across placental circulation is interrupted abruptly [18]. Neonatal abstinence syndrome (NAS) is mainly related to opioids. It appears in 40–70% of children born from heroin-addicted mothers and 68–85% of methadone-maintenance mothers [19–21]. Benzodiazepines have been also related to NAS [22]. It is not well established the presence of NAS in alcohol, nicotine, cannabis or cocaine exposure; however, some of them may induce neurobehavioral or autonomic system disturbances in the newborn (poor neonatal adaptation syndrome) that could may be confused with NAS. Some of these disturbances are tone alteration (e.g., nicotine exposition), tremors (e.g., marijuana) or low alert and poor quality of movement (e.g., cocaine or methamphetamine). They may be related to a hyperserotoninergic state. True NAS symptoms include irritability, tremors, poor feeding, vomiting, diarrhea, sneezing, yawning, excessive sweating or fever. Seizures may appear in 2–11% of cases (more frequent with methadone). Thirty percent of children with NAS present an abnormal electroencephalograph. Most scales for evaluation of NAS (like the Finnegan scale) were primarily conceived for opioid withdrawal. Usually symptoms start after a latency period (24–48 h) or later (2–6 days of life) and are more severe in the case of methadone exposure. Symptoms may last up to 6 months after birth.

Several protocols have been developed to treat NAS depending on the hospital and country. The

American Academy of Pediatrics recommends mechanism-directed therapy (treat opioid withdrawal with an opioid). As the first-line therapy, methadone and morphine are usually employed. Phenobarbital is used in polydrug consumption. Clonidine and oral lorazepam in case of benzodiazepine exposition are possible therapies. Treatment should be gradually tapered, and severe NAS can cause long hospital stays. Nonpharmacologic interventions and hypercaloric diets have been documented as alternative treatments in neonates. Breastfeeding should be encouraged in low-dose methadone mothers (20 mg or less), because NAS interferes with mother–child bonding. Prevention of NAS with an adequate treatment of the dependant mother is the most important aspect. Methadone is still the gold standard to treat opioid-dependence, but buprenorphine has similar effects and some studies suggest that it causes fewer withdrawal symptoms [23,24]. New pharmacotherapeutic strategies for the treatment of opioid dependence are targeting serotonergic system, opioid immunotherapeutics (vaccines) and pharmacogenomics.

Besides NAS, short-term effects may include congenital anomalies (e.g., alcohol, tobacco) and low birth weight (e.g., alcohol, nicotine, methamphetamines, heroin, cocaine). Nevertheless, long-term growth may only be affected in alcohol exposed children [16].

Long-term effects in children

Most drugs have been related to long-term effects in children neurodevelopment. This is supported by the fact that drug exposure affects the structure, function and connectivity of the developing brain. Animal models and neuroimaging studies (functional magnetic resonance imaging) demonstrated alteration on brain structures (hippocampus, white and gray matter) and functions through impaired neurotransmission (dopamine transporter, serotonergic synaptic function, monoamine oxidase pathways). Although there is little evidence to support an effect on overall cognition (with the exception of alcohol and FASD, the main presentable cause of mental disability), several studies have documented effects on specific areas of executive function such as memory, attention, language and learning skills, as well as behavioral problems in children exposed to drugs as compared with controls matched for age and socioeconomic status [25,26]. Sometimes these deficits are subtle, in the form of soft learning disabilities. A recent review conducted on cocaine-exposed school-aged children found a significant negative impact in sustained attention and self-regulated behavior, and little impairment in intelligence quotient (IQ), academic achievement and language functioning [27]. On the other hand, higher rates of substance use and dependence have been observed in the offspring of mothers who smoked or consumed cocaine or other drugs during pregnancy [28]. Alteration in the development of brain areas related to reward processing (e.g., ventral striatum) in tobacco exposition or disrupted oxytocin signaling in case of cocaine exposure have been associated with transgenerational influences of drug abuse and highlight potential epigenetic factors [29].

Behavior and cognition impairments are often caused by the troublesome home environment that goes along with drug abuse (e.g., maternal psychopathology, social isolation, neglect or abuse, domestic violence or inadequate care giving). The improvement of child's environment may contribute to resilient outcomes for these infants [30].

Specimens for *in utero* drug exposure

The analytical determination of drugs and/or metabolites in different biological matrices offers an objective measure of drug exposure during pregnancy. The interpretation of these analytical data tries to answer when the exposure happened during pregnancy, for how long and what amount of drugs were consumed by the mother. The data interpretation also may correlate these concentrations determined in the different matrices with the clinical outcomes.

The biological specimens to investigate *in utero* drug exposure can be from the mother (urine, blood/plasma, oral fluid, sweat, hair), from the newborn (urine, meconium, hair) and from the maternal–fetal unit (placenta, umbilical cord). Maternal monitoring is not a direct marker of *in utero* drug exposure, and in the USA, there are some states that require a newborn specimen as evidence of child abuse [31]. However, this monitoring allows the clinician to be aware of the problem before delivery and to take treatment decisions in advance. Among the different maternal matrices, hair shows the longest window of detection that may be several months. The detection time frame in blood/plasma and oral fluid is hours, in urine days and in sweat a week (depending on the time the patch is worn). Specimens from the newborns and from the fetal–maternal unit show the actual *in utero* exposure to these substances. The specimens most extensively studied are the newborn urine, hair and meconium [11–14]. Drug detection in urine is analytically easy, but it only reflects drug exposure in the last days and its collection can be difficult. The adhesive for the collection bag causes skin irritation and frequently fails to adhere [11]. In the case of hair and meconium, although their analysis is more complex than urine, they show a longer window of detection. Neonatal hair detects drug exposure in the last trimester of pregnancy, and meconium mainly the third and maybe the second

Key term

Method validation: Process of establishing the performance characteristics and limitations of confirmation and screening procedures.

trimester [32–36]. Recently, placenta and umbilical cord have been used successfully as alternative matrices to detect *in utero* drug exposure [32,37–47].

The analytical methods for the determination of alcohol, tobacco and drugs of abuse in the maternal urine, blood/plasma, oral fluid, sweat and neonatal urine are the same methods that are applied in general forensic and clinical toxicology, and therefore, these are not part of the present review. We focused on screening and confirmation methods for drug detection in meconium, placenta, umbilical cord, newborn hair and other alternative matrices (amniotic fluid, nails). The confirmation methods should be fully validated in a given biological matrix, applied to real specimens as proof of concept and fulfill the confirmation criteria [48–50].

In the present review, we included the analytical methods that described the validation performed. Ideally, the method validation should evaluate the following parameters: linearity, bias and precision, calibration model, carryover, interferences, ionization suppression/enhancement (in LC–MS/MS methods), LOD and LOQ, dilution integrity and stability [50]. Linearity (R^2) should be evaluated with ≥6 nonzero calibrators on 5 days. Acceptable linearity is achieved when $R^2 \geq 0.99$ and calibrators quantified within ±20%. LOD and LOQ should be determined with decreasing concentrations of drug-fortified samples in triplicate on three different days (n = 9). LOD is the lowest concentration with acceptable chromatography, signal/noise ratio ≥3, with analytes identified according to the confirmation criteria for this technique. LOQ is the lowest concentrations that met LOD criteria and a signal/noise ratio of at least 10, and bias and precision within ±20%. Assay bias and imprecision should be determined at three concentrations (low, medium and high QCs) in triplicate over 5 days (n = 15). Ion suppression/enhancement should be assessed by comparing analyte peak areas in 10 different blank samples fortified with analyte and internal standard after extraction (Set 1), to peak areas of neat samples (n = 6) at the same concentrations (Set 2). Interferences from endogenous matrix components should be investigated by analyzing samples from 10 individuals without the addition of internal standard. Exogenous interferences should include illicit and licit drugs and metabolites that could be present in the specimen but not included in the method. These interferences are analyzed by fortifying neat samples with high interferences concentrations. Interferences as considered insignificant if analytes of interest were <LOD. Lack of carryover is demonstrated by injecting triplicate internal standard-fortified blank samples after a sample fortified at or above the upper limit of linearity. Carryover was considered negligible if the measured concentration was <LOD. Dilution integrity experiments should be performed with blank matrix. Diluted samples should quantify within ± 20% of the target concentration. Autosampler stability should be investigated by reinjecting low- and high-QC samples stored 48 h at 4°C on the autosampler (n = 3) and calculating results against the original calibration curve. Short-term stability should be evaluated with samples fortified at low- and high-QC concentration and stored for 24 h at room temperature (n = 3), 72 h at 4°C (n = 3) and -20°C (n = 3), and after three freeze–thaw cycles (n = 3). Stability is considered acceptable if QC samples quantified within ±20% of freshly prepared QC samples (n = 3).

Meconium

Meconium has become the gold standard for drug exposure detection in newborns. Meconium is the first stool of an infant, odorless, with dark color and it is composed of mucopolysaccharides, water, bile, salts, bile acids, epithelial cells and other lipids. This makes meconium a complex and viscous matrix from an analytical point of view. Its formation starts around the twelfth week of gestation and accumulates until birth. During the last trimester (week 28–40), its formation increases, and 75% of the meconium is produced during the last 8 weeks of pregnancy [51]. Therefore, the results may provide an overview of the last 2–3 months before birth [52]. Meconium expulsion is sequential and may take up to 5 days. The expulsion can be delayed in premature infants or infants with gastrointestinal disorders. In some cases, this matrix is unavailable for drug testing because meconium could be passed *in utero*, or it could be not collected inadvertently or intentionally by parents to avoid detection of illicit drugs. Meconium specimens should be frozen after collection, if they are not analyzed immediately after sampling. This is especially critical for the fatty acid ethyl esters (FAEEs) [53].

Table 1 summarizes the confirmation method papers in meconium published from 2007 to 2014. Most of the recently published methods dealt with the determination of the alcohol biomarkers, ethyl-glucuronide (EtG), ethyl-sulfate (EtS) and/or FAEEs [52–60]. Himes *et al.* [53] reported the only method able to analyze EtG, EtS and FAEEs from the same meconium aliquot (Figure 1). In the methods for alcohol biomarkers determination [52–60], meconium homogenization

Table 1. Summary of confirmation methods in meconium published from 2007 to 2014.

Analytes	Sample amount	Linearity (ng/g)	Sample preparation	Sample clean-up	Instrument and detection mode	Chromatography	Ref.
Alcohol							
9 FAEEs EtG EtS	100 mg	FAEEs: 25–50 to 2500–5000; EtG: 5–1000 EtS: 2.5–1000 ng/g	1 ml methanol Homogenization with wooden applicators sticks	FAEEs: SLE+ EtG and EtS: SPE Evolute-AX cartridges	LC–MS/MS; FAEEs: ESI+, MRM; EtG and EtS: ESI -, MRM	FAEEs: Poroshell C8 column; MP: 0.1% formic acid in water and methanol; EtG and EtS: Kinetex XB-C18 column; MP: 0.1% formic acid in water and methanol	[53]
6 FAEEs	50 mg	50–100 to 1000	2 ml n-heptane: acetone (5:2) Homogenization vortexing	HS-SPME 65μm, PDMS/DVB	GC–MS, SIM	HP5–MS capillary column	[52]
3 FAEEs	500 mg	500–5000	1 ml water + 12 ml hexane Microwave assisted extraction		GC–MS, FullScan/ SIM	HP5–MS capillary column	[54]
4 FAEEs	500 mg	50–1000	5 ml n-heptane: acetone (5:2) Homogenization vortexing	HS-SPME 65μm, PDMS/DVB	GC–MS, FullScan	FactorFour capillary column	[55]
EtG	200 mg	50–1200	0.2 ml water + 1 ml acetonitrile Ultrasonication	SPE Varian-Aminopropyl cartridges	LC–MS/MS, ESI-, MRM	Luna HILIC column; MP: Ammonium acetate 2 mM in water and acetonitrile	[56]
8 FAEEs	100 mg	10–150 to 1000	2 ml water + 10 mg NaCl Homogenization vortexing	HS-SPME 100μm, PDMS	GC–MS, FullScan	HP5MS capillary column	[57]

3-OH-COT: 3-Hydroxycotinine; 6AM: 6-Acetylmorphine; 11-nor-9-carboxy-THC; 11-OH-THC: 11-hydroxy-THC; AEME: Anhydroecgonine methyl ester; AMP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylecgonine; BUP: Buprenorphine; BSTFA-TMCS: N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane; CBN: Cannabinol; CE: Cocaethylene; COC: Cocaine; COD: Codeine; COT: Cotinine; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EME: Ecgonine methyl ester; ESI: Electrospray ionization; EtG: Ethyl-glucuronide; EtS: Ethyl-sulfate; FAEEs: Fatty acid ethyl esters; HMA: 4-Hydroxy-3-methoxyamphetamine; HMMA: 4-Hydroxy-3-methoxymethamphetamine; HS-SPME: Headspace-solid phase microextraction; HYC: Hydrocodone; HYM: Hydromorphone; M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; MAMP: Methamphetamine; MDA: 3,4-Methylenedioxyamphetamine; MDEA: 3,4-Methylenedioxyethylamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; MOR: Morphine; MRM: Multiple reaction monitoring; MTD: Methadone; MSTFA: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NBUP: Norbuprenorphine; NIC: Nicotine; NORCOT: Norcotinine; NORNIC: Nornicotine; NOREPH: Norephedrine; OH-BE: Hydroxyl-benzoylecgonine; OXYC: Oxycodone; pOHAMP: p-Hydroxyamphetamine; pOHMAMP: p-hydroxy-methaphetamine; PFP: 2,2,3,3,3-Pentafluoro-1-propanol; PFPA: Pentafluoropropionic anhydride; SIM: Single ion monitoring; SLE: Supported liquid extraction; SPE: Solid-phase extraction; THC: Δ9-Tetrahydrocannabinol; THCCOOH: Trans-3′-hydroxycotinine.

Table 1. Summary of confirmation methods in meconium published from 2007 to 2014 (cont).

Analytes	Sample amount	Linearity (ng/g)	Sample preparation	Sample clean-up	Instrument and detection mode	Chromatography	Ref.
9 FAEEs	500 mg	50–500	0.25 ml water + 0.5 ml acetone Vortex 1 min + 5 ml hexane Horizontal shaker	SPE Sep-Pak Aminopropyl cartridges	LC–MS/MS, ESI+, MRM	XBridge C8 column; MP: 0.1% formic acid in 90% acetonitrile	[58]
EtG	20 mg	30	0.5 ml water + 0.5 ml methanol Homogenization vortexing	Filtration	LC–MS/MS, ESI-, MRM	Hypercarb column; MP: 0.1% formic acid in 8% acetonitrile	[59]
EtG EtS	200 mg	EtG: 5–500; EtS: 1–500	1 ml acetonitrile Ultrasonication	SPE Aminopropyl cartridges	LC–MS/MS, ESI-, MRM	Chrompack Inertsil ODS-3 column; MP: 0.1% formic acid in water and acetonitrile	[60]
Cannabis							
THC, THC-COOH, 11-OH-THC and CBN	250 mg	10–15 to 500	0.5 ml methanol Homogenization with wooden applicator sticks Enzymatic and basic hydrolysis	SPE Cerex Polycrom THC cartridges	2x GC–MS, SIM	Derivatization: BSTFA-TMCS Columns: ZB-50 and DB1–MS	[61]
THC-COOH and 11-OH-THC	1 g	10 to 500–1000	3 ml methanol Tissue homogenizer Enzymatic hydrolysis	SPE Cerex Polycrom THC cartridges	2xGC–MS, SIM	Derivatization: MSTFA Columns: DB-5MS and DB-17MS	[62]
Benzodiazepines							
α-OH-alprazolam, α-OH-ethylflurazepam, α-OH-triazolam, alprazolam, desalkylflurazepam, diazepam, lorazepam, midazolam, nordiazepam, oxazepam, temazepam, clonazepam and 7-aminoclonazepam	1 g	20–5000	3 ml methanol Tissue homogenizer Enzimatic hydrolysis	SPE Trace-B cartridges	LC–MS/MS, ESI+, MRM	XTerra MS C18; MP: 55% acetonitrile, 40% water, 5% 100 mM ammonium formate	[63]

3-OH-COT: 3-Hydroxycotinine; 6AM: 6-Acetylmorphine; 11-nor-9-carboxy-THC; 11-OH-THC: 11-hydroxy-THC; AEME: Anhydroecgonine methyl ester; AMP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylecgonine; BUP: Buprenorphine; BSTFA-TMCS: N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane; CBN: Cannabinol; CE: Cocaethylene; COC: Cocaine; COD: Codeine; COT: Cotinine; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EME: Ecgonine methyl ester; ESI: Electrospray ionization; EtG: Ethyl-glucuronide; EtS: Ethyl-sulfate; FAEEs: Fatty acid ethyl esters; HMA: 4-Hydroxy-3-methoxyamphetamine; HMMA: 4-Hydroxy-3-methoxymethamphetamine; HS-SPME: Headspace-solid phase microextraction; HYC: Hydrocodone; HYM: Hydromorphone; M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; MAMP: Methamphetamine; MDA: 3,4-Methylenedioxyamphetamine; MDEA: 3,4-Methylenedioxyethylamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; MOR: Morphine; MRM: Multiple reaction monitoring; MTD: Methadone; MSTFA: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NBUP: Norbuprenorphine; NIC: Nicotine; NORCOT: Norcotinine; NORNIC: Nornicotine; NOREPH: Norephedrine; OH-BE: Hydroxyl-benzoylecgonine; OXYC: Oxycodone; pOHAMP: p-Hydroxyamphetamine; pOHMAMP: p-hydroxy-methaphetamine; PFP: 2,2,3,3,3-Pentafluoro-1-propanol; PFPA: Pentafluoropropionic anhydride; SIM: Single ion monitoring; SLE: Supported liquid extraction; SPE: Solid-phase extraction; THC: Δ9-Tetrahydrocannabinol; THCCOOH: Trans-3′-hydroxycotinine.

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Analytes	Sample amount	Linearity (ng/g)	Sample preparation	Sample clean-up	Instrument and detection mode	Chromatography	Ref.
Amphetamines							
AMP, MAMP, NOREPH, MDA, MDMA, MDEA, HMMA, HMA, pOHAMP and pOHMAMP	1 g	1.25–40 to 2500 ng/g (10 µl injection volume); 125–250 to 10,000 ng/g (1 µl injection volume)	3 ml 17 mM HCl in methanol Ultrasonic homogenization and ultrasonic bath	SPE Strata XC cartridges	LC–MS/MS, APCI+, MRM	Synergi Polar; MP: 10 mM ammonium acetate containing 0.01% (v/v) formic acid and acetonitrile	[64]
AMP and MAMP	N/A	75–3000	3 volumes methanol: water (50:50) ultrasonication	SLE ISOLUTE HM-N	GC–MS, SIM	Derivatization: 4-carbethoxyhexafluorobutyryl HP-5MS column	[65]
Buprenorphine							
BUP and NBUP	250 mg	20–2000	2 ml 1.1 M sodium acetate buffer Ultrasonic disruption and ultrasonic bath	SPE Clean Screen ZSDAU020 cartridges	LC–MS, APCI+, FullScan/ Product ion scan	Synergi Polar; MP: 20 mM ammonium acetate buffer with 0.05% formic acid at pH 4.6 and acetonitrile	[66]
Cocaine							
COC, AEME, BE, EME and CE	500 mg	20–30 to 1500	ASE (2.4 g diatomaceous Earth, phosphate buffer 0.1M pH 6)	SPE Bon Elut Certify cartridges	GC–MS, FullScan/ SIM	Derivatization: PFPA and PFPHP-5 MS capillary column	[67]
Opiates							
COD, MOR, HYC, HYM, OXY and 6AM	1 g	2–2500	3 ml methanol Tissue homogenizer	SPE Trace-B cartridges	LC–MS/MS, ESI+, MRM	Nova-Pak CN HP column; MP: 85% 2mM ammonium formate buffer at pH 3.0 and 15% acetonitrile	[68]

3-OH-COT: 3-Hydroxycotinine; 6AM: 6-Acetylmorphine; 11-nor-9-carboxy-THC; 11-OH-THC: 11-hydroxy-THC; AEME: Anhydroecgonine methyl ester; AMP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylecgonine; BUP: Buprenorphine; BSTFA-TMCS: N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane; CBN: Cannabinol; CE: Cocaethylene; COC: Cocaine; COD: Codeine; COT: Cotinine; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EME: Ecgonine methyl ester; ESI: Electrospray ionization; EtG: Ethyl-glucuronide; EtS: Ethyl-sulfate; FAEEs: Fatty acid ethyl esters; HMA: 4-Hydroxy-3-methoxyamphetamine; HMMA: 4-Hydroxy-3-methoxymethamphetamine; HS-SPME: Headspace-solid phase microextraction; HYC: Hydrocodone; HYM: Hydromorphone; M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; MAMP: Methamphetamine; MDA: 3,4-Methylenedioxyamphetamine; MDEA: 3,4-Methylenedioxyethylamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; MOR: Morphine; MRM: Multiple reaction monitoring; MTD: Methadone; MSTFA: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NBUP: Norbuprenorphine; NIC: Nicotine; NORCOT: Norcotinine; NORNIC: Nornicotine; NOREPH: Norephedrine; OH-BE: Hydroxyl-benzoylecgonine; OXYC: Oxycodone; pOHAMP: p-Hydroxyamphetamine; pOHMAMP: p-hydroxy-methaphetamine; PFP: 2,2,3,3,3-Pentafluoro-1-propanol; PFPA: Pentafluoropropionic anhydride; SIM: Single ion monitoring; SLE: Supported liquid extraction; SPE: Solid-phase extraction; THC: Δ9-Tetrahydrocannabinol; THCCOOH: Trans-3′-hydroxycotinine.

Table 1. Summary of confirmation methods in meconium published from 2007 to 2014 (cont).

Analytes	Sample amount	Linearity (ng/g)	Sample preparation	Sample clean-up	Instrument and detection mode	Chromatography	Ref.
Tobacco							
NIC, COT, 3-OH-COT, NORNIC and anabasine	0.25 g	2–4 to 50–100	1 ml methanol Bullet blender	SPE Trace-B cartridges	LC–MS/MS, ESI+, MRM	Atlantis HILIC; MP: 85% acetonitrile, 10% water and 5% 100 mM ammonium formate buffer at pH 3	[69]
NIC, COT, 3-OH-COT, NORNIC and NORCOT	500 mg	1.25–5 to 500	2 ml methanol +0.01% formic acid Ultrasonication Enzimatic hydrolysis	SPE Clean Screen DAU020 cartridges	LC–MS/MS, APCI+, MRM	Synergi Polar; MP: 0.01M ammonium acetate, pH 6.8 and acetonitrile with 0.01% formic acid	[70]
Multiple drug groups							
NIC, COT, COC, BE, CE and AEME	300 mg	20–900; 100–1000 (nicotine)	2 ml methanol Horizontal shaker	SPE TIPS DPX	GC–MS, SIM	Derivatization: MSTFA HP-5MS column	[71]
MOR, M3G, M6G, COD, 6AM, AMP, MAMP, MDA, MDMA, COC, BE, EME, OH-BE, MTD and EDDP	500 mg	5–500	2 ml methanol + 0.01% formic acid Shaker	SPE OASIS MCX cartridges	LC–MS/MS, ESI+, MRM	Atlantis T3; MP: acetonitrile and formic acid 0.1%	[32]
COC, BE, COD, MOR and 6AM	500 mg	20–1000	4 ml methanol Shaker	SPE Bond Elut cartridges	GC–MS, SIM	Derivatization: BSTFA/TMCS HP-5MS column	[72]
AMP, MAMP, pOHMAMP, COC, BE, CE, OH-BE, NIC, COT, 3-OH-COT, 6AM, MOR, COD, HYM, HYC, OXYC, MTD, EDDP, BUP and NBUP	250 mg	1–5 to 500	1 ml methanol +0.01% formic acid Shaker	SPE Clean Screen DAU020 cartridges	LC–MS/MS, ESI+, MRM	Synergi Hydro (2 chromatographic runs); MP: 1 mM ammonium formate, pH 3.4 and acetonitrile	[73]

3-OH-COT: 3-Hydroxycotinine; 6AM: 6-Acetylmorphine; 11-nor-9-carboxy-THC; 11-OH-THC: 11-hydroxy-THC; AEME: Anhydroecgonine methyl ester; AMP: Amphetamine; APCI: Atmospheric pressure chemical ionization; BE: Benzoylecgonine; BUP: Buprenorphine; BSTFA-TMCS: N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane; CBN: Cannabinol; CE: Cocaethylene; COC: Cocaine; COD: Codeine; COT: Cotinine; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EME: Ecgonine methyl ester; ESI: Electrospray ionization; EtG: Ethyl-glucuronide; EtS: Ethyl-sulfate; FAEEs: Fatty acid ethyl esters; HMA: 4-Hydroxy-3-methoxyamphetamine; HMMA: 4-Hydroxy-3-methoxymethamphetamine; HS-SPME: Headspace-solid phase microextraction; HYC: Hydrocodone; HYM: Hydromorphone; M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; MAMP: Methamphetamine; MDA: 3,4-Methylenedioxyamphetamine; MDEA: 3,4-Methylenedioxyethylamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; MOR: Morphine; MRM: Multiple reaction monitoring; MTD: Methadone; MSTFA: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; NBUP: Norbuprenorphine; NIC: Nicotine; NORCOT: Norcotinine; NORNIC: Nornicotine; NOREPH: Norephedrine; OH-BE: Hydroxyl-benzoylecgonine; OXYC: Oxycodone; pOHAMP: p-Hydroxyamphetamine; pOHMAMP: p-hydroxy-methaphetamine; PFP: 2,2,3,3,3-Pentafluoro-1-propanol; PFPA: Pentafluoropropionic anhydride; SIM: Single ion monitoring; SLE: Supported liquid extraction; SPE: Solid-phase extraction; THC: Δ9-Tetrahydrocannabinol; THCCOOH: Trans-3′-hydroxycotinine.

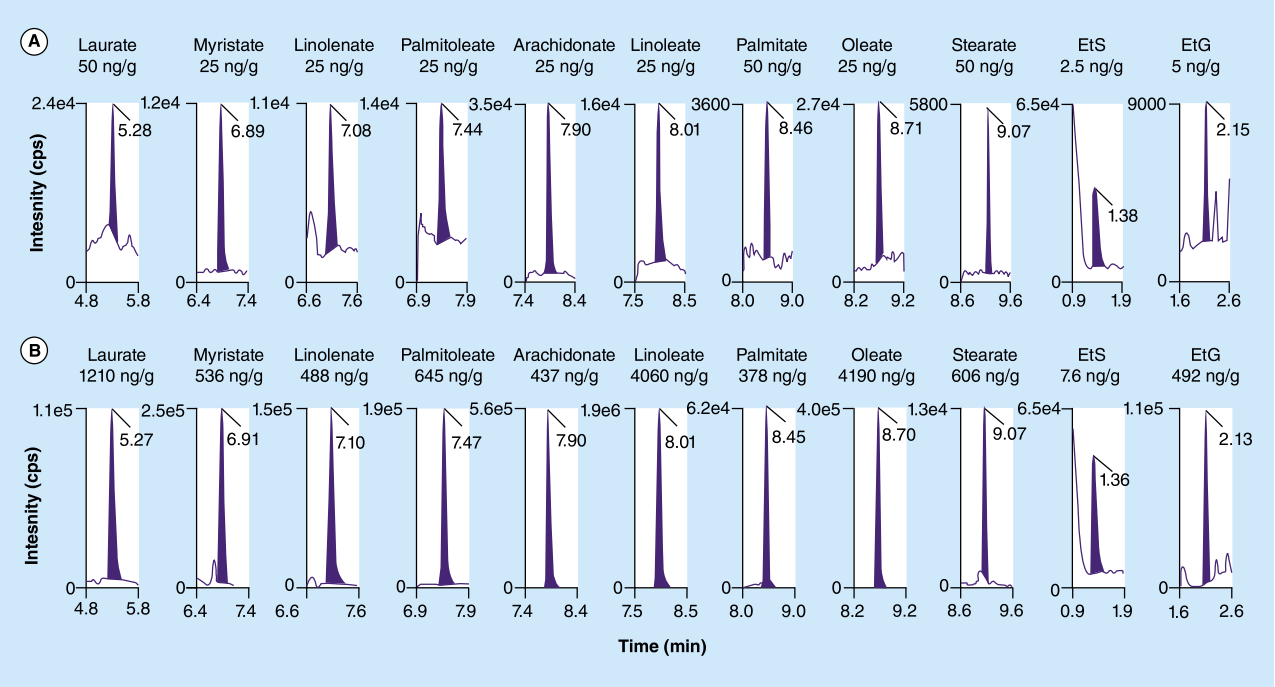

Figure 1. Multiple reaction monitoring chromatograms for alcohol marker quantifier ions. (A) blank meconium fortified at analyte limits of quantification and **(B)** authentic positive meconium specimens with concentrations of alcohol markers listed under the name of each marker.
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was performed manually with wooden applicators [53], mixing with horizontal shaker [58], vortexing [52,55,57,59] or sonicating [56,60], in the presence of organic solvents [52,53,55,60], aqueous and organic mixtures [56,58,59] or aqueous solutions [57]. For EtG and EtS, the sample clean-up performed was SPE with anion exchange cartridges [53] or with aminopropyl cartridges [56,60], or just a simple sample filtration [59]. In the case of the FAEEs, several authors used head space solid-phase microextraction (HS-SPME) [52,55,57], but also SPE with aminopropyl cartridges [58] and supported liquid extraction (SLE) [53]. Carbacos *et al.* [54] performed simultaneously the sample homogenization and clean-up by microwave assisted extraction. EtG and EtS were determined by LC–MS/MS, in electrospray negative mode (ESI-), monitoring 2 transitions per analyte [53,56,59,60]. The chromatographic separation was performed in reverse phase columns [53,60]. However, due to the high polarity of EtG and EtS, some methods used hydrophilic interaction chromatographic columns (HILIC) [56], which are a variant of normal phase LC, and Hypercarb columns [59], which are porous graphic carbon columns with different retention and selectivity than reverse phase columns. FAEEs were determined by LC–MS/MS using reverse phase and ESI in positive mode [53,58] and by GC–MS [52,54,55,57]. Among the different methods for the determination of alcohol biomarkers in meconium, the method developed by Himes *et al.* [53] is the most interesting. The authors were able to detect EtG, EtS and FAEEs from the same meconium aliquot, using low amount of sample (100mg) and achieving good sensitivity. Due to the different chemical properties, it is extremely difficult to extract EtG, EtS and FAEEs simultaneously. Himes *et al.* [53] approach was to perform a common homogenization of the sample, then to split the homogenate and perform two different extractions and LC–MS/MS methods one for EtG and EtS and another for FAEEs.

Gray *et al.* [61] and Marin *et al.* [62] published methods for the determination of cannabinoids in meconium. Marin *et al.* [62] only analyzed delta-9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-THC (THCCOOH), while Gray *et al.* [61] also included 11-hydroxy-THC (11-OH-THC) and cannabinol (CBN). Both groups achieved the same sensitivity (LOQ 10 ng/g), but Gray *et al.* [61] used less amount

Key term

LC–MS/MS: Analytical technique that combines the separation capabilities of LC with the m/z analysis of LC. The mass spectrometer can be just one (LC–MS) or in tandem (LC–MS/MS). This technique is sensitive, specific and versatile.

of sample (250 mg) than Marin *et al.* [62] (1 g). Gray *et al.* [61] and Marin *et al.* [62] performed meconium homogenization in methanol, manually [61] or mechanically [62]. Marin *et al.* [62] applied an enzymatic hydrolysis, but Gray *et al.* [61] employed both basic and enzymatic hydrolysis. According to the authors [61], the hydrolysis efficiencies were analyte-dependent; delta9-tetrahydrocannabinol-glucuronide (THC-glucuronide) was effectively cleaved by enzyme, but not base. Conversely, 11-nor-9-carboxy-THC-glucuronide (THCCOOH-glucuronide) was most sensitive to alkaline hydrolysis. The sample clean-up was performed with Cerex Polycrom THC cartridges and the detection by 2-dimensional GC–MS instruments [61,62].

Marin *et al.* [63] published a method for the determination of 13 benzodiazepines and metabolites. Benzodiazepines are psychoactive drugs normally used in the treatment of anxiety disorders, and chemically most of them are neutral-acidic compounds. The method used 1 g meconium and the LOQ was 20 ng/g. The meconium homogenization was performed in methanol with a tissue homogenizer, and the samples were hydrolyzed. After evaporation of the supernatant, the samples were extracted by SPE dual-mode resin cartridges (reverse phase and cation exchange). The chromatographic separation was performed using a reverse-phase column, and the analytes were detected using ESI in positive mode.

Several confirmation methods were published for the determination of basic drugs including only one group of compounds, such as amphetamines [64,65], buprenorphine [66], cocaine [67], opiates [68] or tobacco biomarkers [69,70], or including multiple drug groups [32,71–73]. Multi-analyte procedures are more interesting than methods that include only one group of compounds because they save specimen, time and cost if different drug groups have to be analyzed in the same specimen. The multi-analyte methods [32,71–73] employed similar or less amount of specimen than the one group methods (250–500 mg), and they achieved comparable sensitivity (LOQ 1–20 ng/g). In these methods, meconium samples were homogenized in methanol [68,69,71], acidified methanol [32,64,70], methanol and water mixture [65] or aqueous buffer [66] by sonication [64–66,70], tissue homogenizer [68], bullet blender [69] or shaker [32,71]. Mantovani *et al.* [67] employed a different approach for the initial extraction of cocaine and metabolites from meconium samples, the accelerated solvent extraction (ASE). ASE is an automated technique accomplished with liquid solvents under elevated temperature (120°C) and pressure (1500 psi) aiming at faster (2 min) and more efficient analyte extraction [67]. Most of the authors performed the sample clean-up by SPE, using reverse phase, cation exchange [32,64] or dual mode cartridges [66–70,72,73]. Bordin *et al.* [71] employed cation exchange disposable pipette extraction tips, which allowed them to reduce extraction time and solvent consumption. Gunn *et al.* employed SLE [65].

With regard to screening methods, several articles were published evaluating and/or comparing commercially available immunoassays [74–76], or screening methods based on LC–MS/MS and high-resolution MS (HRMS) [77]. Pichini *et al.* [74] evaluated an ELISA for the identification of EtG in meconium. The assay used 0.25 g meconium, and the cut-off was 200 ng/g. Marin *et al.* [75] compared ELISA and biochip microarrays for the detection of cannabinoids, amphetamine, methamphetamine, methadone, benzoylecgonine (BE), phencyclidine (PCP), barbiturates, benzodiazepines and opioids, in 0.25 g meconium. Both techniques compared well, but the specificity of the biochip assay was slightly better for amphetamines and cocaine. The same group [76] compared the enzyme multiplied immunoassay technique and ELISA for the detection of cannabinoids, amphetamine, methamphetamine, methadone (MTD), propoxyphene, BE, PCP, barbiturates, benzodiazepinese, opioids and oxycodone. ELISA offered lower cut-offs, shorter processing time and employed less amount of sample (0.25 vs 0.5 g). Ristimaa *et al.* [77] developed target drug screening methods in meconium by LC–MS/MS and by LC–HRMS, using TOF. The two qualitative methods developed by LC–MS/MS detected four amphetamines and eight opioids at 0.2–6 ng/g cut-off (method 1) and THC-COOH at 20 ng/g cut-off (method 2). The MS operated in ESI positive mode, and two transitions were monitored for each analyte. The LC–TOFMS identification was based on an in-house mass database containing 869 molecular formulae and monoisotopic masses of illicit and licit drugs and their metabolites, and retention times for about half of these compounds.

Placenta

The placenta is the interface between maternal and fetal blood. Its basic structure is formed at 4 weeks of pregnancy. At term, placenta weights around 500 g, and it has 20 cm diameter by 3 cm thickness. Endogenous and exogenous compounds are exchanged between the mother and the embryo or fetus through the placenta that also has important endocrine and metabolic functions. Drugs cross the placenta mainly by passive diffusion from the mother to the embryo/fetus with the extent of transfer dependent on the drug's physicochemical properties (liposolubility, size, protein binding) [32]. Some *in vitro* studies suggested that placenta could act as a depot for drugs, depending on the affinity of the analytes for this tissue [78,79]. Placenta specimens should be stored at -20°C after collection [80].

Placenta has been used as alternative matrix to monitor drugs of abuse during pregnancy [32,42–47]. The main advantages of the placenta are its easy and noninvasive collection, the abundant amount of specimen, readily available at delivery and the fact that it is considered a waste product. The window of detection is unknown. However, a recent study suggested that this could be similar to meconium but with lower concentrations and a different drug/metabolite profile [32].

Several confirmation methods have been published recently for the determination of alcohol biomarkers [81,82], buprenorphine [83] and multiple drug groups [80,84,85] in placenta. Matlow *et al.* [80] determined EtG in 1 g of placenta achieving 5 ng/g LOQ. The tissue was homogenized in water with formic acid with a tissue blender. Further clean-up was performed by SPE, followed by HS-SPME. The compound was analyzed by GC–MS. Morini *et al.* [82] determined EtG and EtS by LC–MS/MS. The authors used 0.5 g of placenta and achieved 5 ng/g LOQ. Morini *et al.* [82] performed a simpler and faster sample preparation than Matlow *et al.* [81]. In Morini *et al.*'s method [82], the placenta tissue was pretreated vortexing in acetonitrile, and a simple sample dilution was performed before injecting into the LC–MS/MS.

Concheiro *et al.* [83] determined bupernorphine, and its metabolites, norbuprenorphine and their glucuronides, in 2 g placenta. The tissue was homogenized in water with perchloric acid using a tissue blender, and the supernatant was extracted by cation exchange SPE cartridges. The instrument employed was LC–MS.

Three methods [80,84,85] allowed the simultaneous determination of multiple drug groups (Figure 2). All these methods used the same amount of specimen (1 g) and achieved similar sensitivity (1–5 ng/g). de Castro *et al.* [80] detected 16 different compounds, while Joya *et al.* [84] and a previous method by de Castro *et al.* [85] detected 11 and seven compounds, respectively. Tissue homogenization was performed in aqueous solvent by a tissue homogenizer [80,85] or ultrasonication [84]. All methods [80,84,85] utilized cation exchange SPE for supernatant clean-up. The instrumentation used was LC–MS/MS [80,85] and GC–MS [84]. A summary of these methods is showed in Table 2.

Umbilical cord

The umbilical cord is the conduit between the developing embryo or fetus and the placenta. It contains two umbilical arteries and one umbilical vein surrounded by Wharton's jelly that protects and insulates the umbilical cord vessels. At term, the umbilical cord is about 50 cm long and 2 cm wide [86]. Umbilical cord has been used as alternative matrix to monitor drugs of abuse during pregnancy [32,37–41]. As indicated for the placenta, the main advantages of the umbilical cord are its easy and noninvasive collection, the abundant amount of specimen, readily available at delivery and the fact that it is considered a waste product. A recent study suggested that its window of detection could be similar to meconium but with lower concentrations and different drug/metabolite profile [32]. Umbilical cord specimens should be stored at -20°C after collection [80].

Confirmation methods for tobacco biomarkers [69], buprenorphine and metabolites [87], amphetamines [88] and multiple drug groups [80,89] have been developed in umbilical cord (Table 3). The amount of sample employed ranged from 1 to 2 g, and the LOQ from 0.25 to 10 ng/g. The umbilical cord samples (1–2 g) were homogenized in organic [69,88] or aqueous [80,87,89] solvents using a tissue blender or a bullet blender. In all the published methods, the samples were extracted by SPE using mixed mode [69,88] or cation exchange [80,87,89] cartridges. The analytes were detected by LC–MS/MS using ESI in positive mode [69,80,87–89]. The chromatographic separations were performed in reverse phase [80,88,89], except a method for the determination on tobacco biomarkers that used HILIC column [69].

Chittama *et al.* [90] published a comparison of three different screening methods for cannabinoids in umbilical cord. The target analyte was THC-COOH, and they compared ELISA, GC–MS and LC–TOF–MS. The authors concluded that the GC–MS method provided better sensitivity (cut-off 0.05 ng/g) than ELISA (cut-off 0.1 ng/g) and LC–TOF–MS (cut-off 1 ng/g). Marin *et al.* [91] published a broad screening method of 57 drugs in umbilical cord by LC–TOFMS. The 57 drugs included opioids and antagonists, benzodiazepines and hypnotics, barbiturates, stimulants and PCP. The method employed 1 g of umbilical cord, and the cut-offs ranged from 1 to 40 ng/g. The samples were homogenized (2 ml water 0.1% Trintox-100, bullet blender) and extracted (SLE) before injection into the system.

Hair

Mother hair is the matrix that offers the longest window of detection. Hair grows at an accepted rate of 1 cm/mo [92–94]. If the lock of hair is long enough, and through

Key term

High-resolution MS: Analytical technique that allows the separation of ions differing slightly in *m/z*. The high-resolution mass spectrometers available nowadays are TOF and orbitrap

Alternative matrix: Nonconventional biological material that is employed to study the presence and/or to determine drugs and/or metabolites.

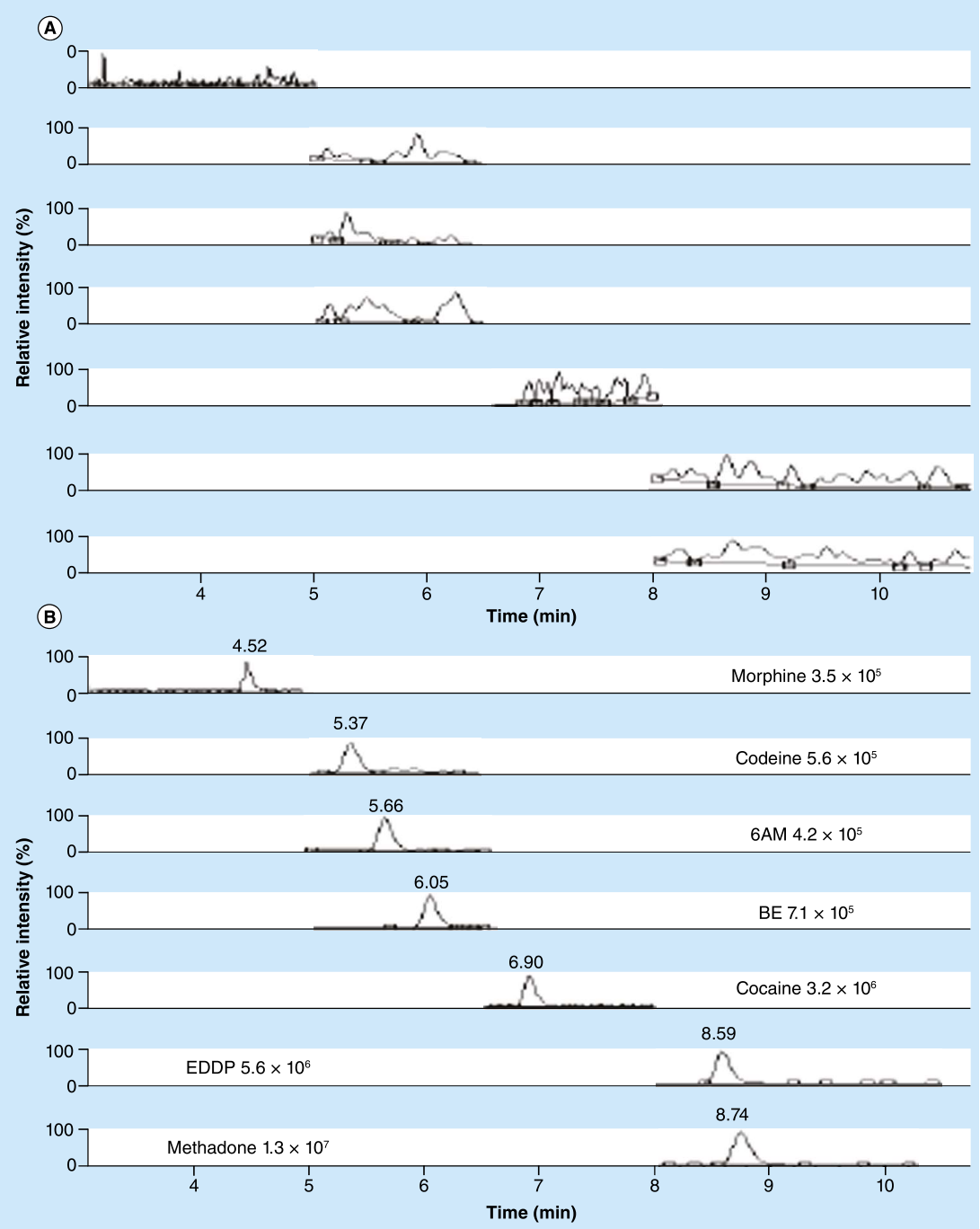

Figure 2. Chromatogram of the quantifier transitions for morphine, codeine, 6-acetylmorphine, benzoylecgonine, cocaine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and methadone. Quantifier transitions in **(A)** extracted blank placenta, **(B)** placenta fortified at the limit of quantification and **(C)** authentic placenta from an opioid-dependent woman maintained on daily methadone with a total cumulative dose of 11760 mg (75 mg methadone the day of delivery). The authentic placenta contained morphine (42.3 ng/g), codeine (2.6 ng/g), cocaine (7.9 ng/g), BE (496.3 ng/g), methadone (1346.3 ng/g) and EDDP (84.7 ng/g) indicating heroin or morphine, cocaine and methadone exposure.
6AM: 6-acetylmorphine; BE: benzoylecgonine; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
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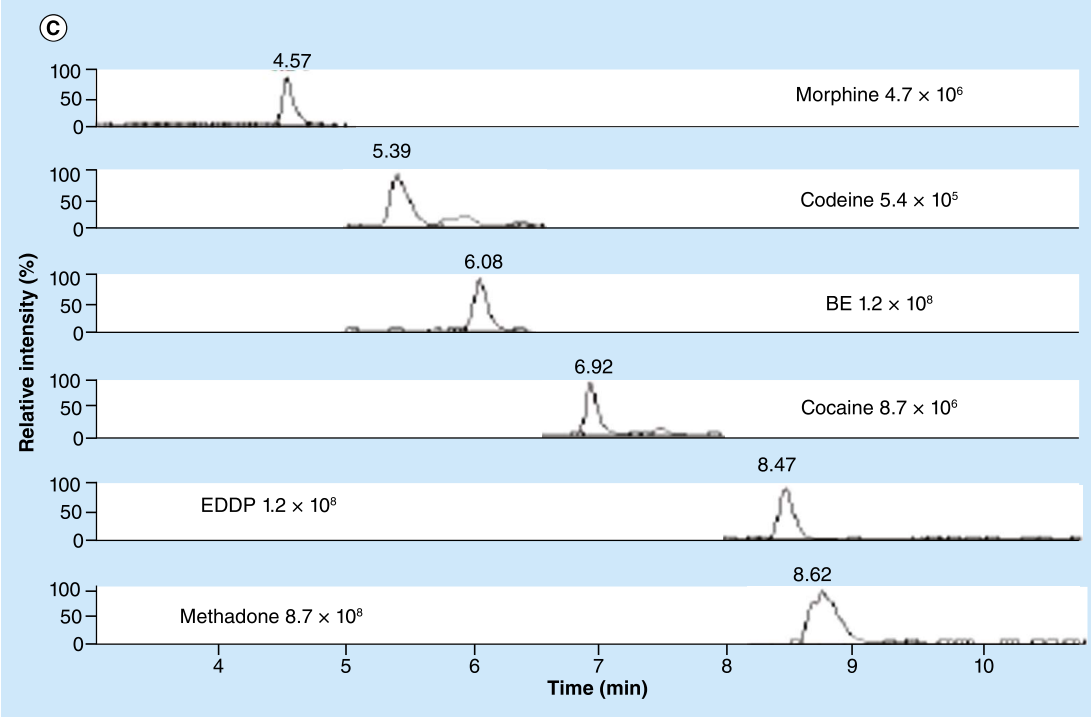

Figure 2. Chromatogram of the quantifier transitions for morphine, codeine, 6-acetylmorphine, benzoylecgonine, cocaine, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine and methadone. Quantifier transitions in **(A)** extracted blank placenta, **(B)** placenta fortified at the limit of quantification and **(C)** authentic placenta from an opioid-dependent woman maintained on daily methadone with a total cumulative dose of 11760 mg (75 mg methadone the day of delivery). The authentic placenta contained morphine (42.3 ng/g), codeine (2.6 ng/g), cocaine (7.9 ng/g), BE (496.3 ng/g), methadone (1346.3 ng/g) and EDDP (84.7 ng/g) indicating heroin or morphine, cocaine and methadone exposure.
6AM: 6-acetylmorphine; BE: benzoylecgonine; EDDP: 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
Reproduced with permission from [85] © Oxford University Press (2014).

the use of segmented analysis, hair can provide a detailed account of drug exposure across the three trimesters of pregnancy. The analysis of 3 cm segments from the root to the tip reflect the mother drug consumption in the first, second and third trimester of pregnancy, respectively [93]. The maternal hair analysis is performed as a regular hair testing. The lock of hair is segmented, washed (to eliminate external contamination), weighted and cut in small pieces or pulverized. After that, incubation takes place to extract the drugs from the matrix, followed by sample clean up. Recently, several reviews have been published about hair analysis [95,96].

Neonatal hair begins to form at approximately 6 months of gestational age; therefore, a positive result indicates use during the last trimester. The main advantages of neonatal hair testing are that it can be collected at any time point within 3 months after birth, until this is replaced by new hair, and the easy storage of the sample at room temperature. However, one complication of testing weeks to months after birth is that the testing may pick up passive drug exposure at home depending on whether washing of the hair sample is performed. As disadvantages we have to highlight that neonatal hair is often not available or in very little amount, or the parents reject its collection because of cosmetic or cultural reasons [11]. Neonatal hair testing is usually performed as regular hair testing; however, there are a couple of considerations that should be taken into account. The first one is the amount of specimen available. As we just said, neonatal hair amount is scarce. Analytical methods should use 10 or less mg. Another important point to evaluate is the necessity to wash or not the hair lock. At birth, drugs in newborn hair could originate from the deposition from fetal blood into the growing hair shaft or from contamination of hair by amniotic fluid. In either case, the only source of drug is from maternal ingestion. Because of this, in the opinion of the authors neonatal hair should not be washed before the analysis if the hair is collected just after birth. Hair samples must be stored in a dry, dark environment at room temperature, away from direct sunlight. Hair samples should not be stored in the refrigerator or freezer, since swelling may occur and drug may be lost [97].

Table 2. Summary of confirmation methods in placenta published from 2007 to 2014.

Analytes	Sample amount (g)	Linearity (ng/g)	Sample preparation	Sample clean-up	Instrument and detection mode	Chromatography	Ref.
Alcohol							
EtG	1	5–500	3 ml water + 150 µl formic acid Vortex Tissue homogenizer	SPE Clean Screen cartridges; HS-SPME	GC–MS (NCI), FullScan	Derivatization: HFBA DB-1HT column	[81]
EtG and EtS	0.5	5–1000	200 µl acetonitrile Vortex	1:10 dilution with water	LC–MS/MS, ESI-, MRM	Chrompack Inertsil ODS-3 column; MP: acetonitrile and 0.1% formic acid	[82]
Buprenorphine							
BUP, NBUP, BUP–Gluc and NBUP–Gluc	2	1–50	8 ml 0.1% perchloric acid in water Tissue homogenizer	SPE Strata-XC cartridges	LC–MS, ESI+, scan/product ion scan	Synergi Polar; MP: acetonitrile and 0.1% formic acid	[83]
Multiple drug groups							
MOR, M3G, M6G, COD, 6AM, COC, BE, EME, OH-BE, CE, AMP, MAMP, MDA, MDMA, MTD and EDDP	1	1–5 to 100–500	5 ml water Tissue homogenizer	SPE OASIS MCX cartridges	LC–MS/MS, ESI+, MRM	Atlantis T3; MP: acetonitrile and 0.1% formic acid	[80]
AMP, MAMP, MDMA, MTD, COC, BE, CE, MOR, THCCOOH, NIC and COT	1	5–500	5 ml 0.1% perchloric acid in water Ultrasonication	SPE Strata X-C cartridges	GC–MS, SIM	Derivatization: MSTFA ZB-5 column	[84]
MTD, EDDP, COC, BE, MOR, COD and 6AM	1 g	2.5–10 to 500–2000 ng/g	5 ml 0.1% perchloric acid in water Tissue homogenizer	SPE Strata X-C cartridges	LC–MS, ESI+, scan/product ion scan	Synergi Polar; MP: acetonitrile and 0.1% formic acid	[85]

6AM: 6-Acetylmorphine; AMP: Amphetamine; BE: Benzoylecgonine; BUP: Buprenorphine; BUP–Gluc: Buprenorphine–glucuronide; CE: Cocaethylene; COC: Cocaine; COD: Codeine; COT: Cotinine; EME: Ecgonine methyl ester; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; ESI: Electrospray ionization; EtG: Ethyl–glucuronide; EtS: Ethyl-sulfate; HS-SPME: Headspace-solid phase microextraction; M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; MAMP: Methamphetamine; MDA: 3,4-Methylenedioxyamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; MOR: Morphine; MRM: Multiple reaction monitoring; MSTFA: *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide; MTD: Methadone; NBUP: Norbuprenorphine; NBUP–Gluc: Norbuprenorphine-glucuronide; NIC: Nicotine; OH-BE: Hydroxyl-benzoylecgonine; SIM: Single ion monitoring; SPE: Solid-phase extraction; THCCOOH: 11-Nor-9-carboxy-Δ9-Tetrahydrocannabinol.

From 2007 to 2014, several method papers have been published for the determination of drugs in neonatal hair [98–102]. Two papers determined antidepressants [98,99], one bupernorphine and its metabolite [100] and two determined multiple drug groups [101,102]. All of them [98–102] employed little amount of hair, 10 or less mg, achieving good sensitivity (LOQ 0.003 to 0.4 ng/mg). All these methods [98,99,101,102] but one [100] washed the hair lock before analysis. The neonatal hair samples were pulverized [98,99] or cut [100–102], and methanol was the solvent most commonly used for incubation [99,101,102]. For sample clean-up, the authors employed LLE [98–100], SPE [101] or a more complex procedure of two consecutive cleaning steps, SPE and HS-SPME [102]. The instrumentation employed was GC–MS [101,102], LC–MS [99,100] and LC–HRMS [98]. These methods are summarized in Table 4.

Other matrices (amniotic fluid, nails)

The amniotic fluid is the protective liquid contained by the sac where the embryo/fetus develops. At the early stages of pregnancy, amniotic fluid consists of a filtrate of maternal blood. During the rest of the pregnancy, amniotic fluid acts as a fetal excretion reservoir, accumulating drugs throughout gestation [11]. Its major disadvantage is its invasive collection that can be harmful to the fetus, unless it is collected at birth [12]. Amniotic fluid specimens should be stored at -20°C after collection [41]. In recent years, only one method was published describing the determination of nicotine and its metabolites in amniotic fluid at birth [103] by reversed-phase HPLC and colorimetric detection at 545 nm.

Newborn nails have been scarcely investigated as alternative matrices for *in utero* drug exposure. Nails are formed during the last trimester of pregnancy

Table 3. Summary of confirmation methods in umbilical cord published from 2007 to 2014.

Analytes	Sample amount (g)	Linearity (ng/g)	Sample preparation	Sample clean-up	Instrument & detection mode	Chromatography	Ref.
Tobacco							
NIC, COT, 3-OH-COT, NORNIC and anabasine	1.5	0.25–0.5 to 6.25–12.5	2 ml methanol Bullet Blender	SPE Trace-B cartridges	LC–MS/MS, ESI+, MRM	Atlantis HILIC; MP: 85% acetonitrile, 10% water and 5% 100 mM ammonium formate buffer at pH 3	[69]
Buprenorphine							
BUP, NBUP, BUP–Gluc and NBUP–Gluc	2	1–50	8 ml 0.1% perchloric acid in water Tissue homogenizer	SPE Strata-XC cartridges	LC–MS, ESI+, scan/product ion scan	Synergi Polar; MP: acetonitrile and 0.1% formic acid	[87]
Amphetamines							
AMP and MAP	1	0.6–100	5.5 ml acetonitrile Tissue homogenizer	SPE Clean Screen ZSDAU020 cartridges	LC–MS/MS, ESI+, MRM	Synergi Hydro; MP: 10mM ammonium acetate and 0.1% formic acid and acetonitrile and 0.1% formic acid	[88]
Multiple drug groups							
MOR, M3G, M6G, COD, 6AM, COC, BE, EME, OH-BE, CE, AMP, MAMP, MDA, MDMA, MTD and EDDP	1	1–5 to 100–500	5 ml water Tissue homogenizer	SPE OASIS MCX cartridges	LC–MS/MS, ESI+, MRM	Atlantis T3; MP: acetonitrile and 0.1% formic acid	[80]
MTD, EDDP, COC, BE, MOR, COD and 6AM	1	2.5–10 to 500–2000	5 ml 0.1% perchloric acid in water Tissue homogenizer	SPE Strata X-C cartridges	LC–MS, ESI+, scan/product ion scan	Synergi Polar; acetonitrile and 0.1% formic acid	[89]

6AM: 6-Acetylmorphine; AMP: Amphetamine; BE: Benzoylecgonine; BUP: Buprenorphine; BUP–Gluc: Buprenorphine-glucuronide; CE: Cocaethylene; COC: Cocaine; COD: Codeine; COT: Cotinine; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EME: Ecgonine methyl ester; ESI: Electrospray ionization; OH-BE: Hydroxyl-benzoylecgonine; M3G: Morphine-3-glucuronide; M6G: Morphine-6-glucuronide; MAMP: Methamphetamine; MDA: 3,4-Methylenedioxyamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; MOR: Morphine; MRM: Multiple reaction monitoring; MTD: Methadone; NBUP: Norbuprenorphine; NBUP–Gluc: Norbuprenorphine-glucuronide; NIC: Nicotine; NORNIC: Nornicotine; 3-OH-COT: Trans-3′-hydroxycotinine; SPE: Solid-phase extraction.

and, because of this, are supposed to reflect the exposure in this period. This specimen should be stored at room temperature protected from the light [104]. Mari *et al.* [105] published a method for the determination of cocaine, BE, morphine, methadone, caffeine, nicotine and cotinine in newborn nail clippings. Nail clippings (10 mg) were washed with methanol, cut with scissors and incubated overnight with hydrochloric acid 0.1 N. Samples were solid phase extracted (mixed mode cartridges), derivatized by N,O-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (BSTFA-1% TMCS) and analyzed by GC–MS. The method's LOQ was 0.025 ng/g for all analytes.

Conclusion

Maternal hair and meconium analysis are the gold standard for the detection of *in utero* drug exposure. Alternative matrices, such as placenta, umbilical cord and nails, could also be employed. All these matrices require a long sample preparation, but they offer the longest window of detection. Sample preparation consists of sample pretreatment, including hair wash/incubation and tissue and meconium homogenization, followed by extraction/clean-up of the analytes. The analytical technique most commonly employed is LC–MS/MS, due to its versatility, sensitivity and specificity. Promising screening methods by LC–HRMS have been published in meconium and umbilical cord. Due to the broad spectrum of covered analytes, this kind of analysis will expand in the future. Analytical methods employed for determination of *in utero* drug exposure have to be fully validated and fulfill the confirmation criteria. High-quality analytical data is essential to perform the interpretation of the results and to study the possible

Table 4. Summary of confirmation methods in newborn hair published from 2007 to 2014.

Analytes	Sample amount (mg)	Linearity (ng/mg)	Hair wash	Sample preparation	Sample clean-up	Instrument and detection mode	Chromatography	Ref.
Antidepressants								
Venlafaxine	2.5	0.2–25	2 × CH_2Cl_2	Simultaneous pulverization+extraction: Steel bullets+ 145 µl water+20 µl acetonitrile +20 µl 1M trifluoroacetic acid		LC–HRMS, ESI+, Full Scan/ Product ion scan	Atlantis T3 column; MP: ammonium acetate 5 mM pH 5 and acetonitrile with 0.1% formic acid	[98]
Citalopram, escitalopram, demethylated metabolites	10	0.025–2	2 × CH_2Cl_2	Hair grinding 2 ml methanol Ultrasonication 3 h	LLE (1 ml water + 200 mg ammonium carbonate+ 5 ml diethylether: dichloromethane, 70:30)	LC–MS, ESI+, Full scan/ Product ion scan	Luna C18 column; MP: water, 0.1% formic acid, ammonium formate 2 mM, pH 3 and sacetonitrile, 0.1% formic acid, ammonium formate 2 mM	[99]
Buprenorphine								
BUP and NBUP	1.6–5.3	0.003–10	No wash	2 M NaOH overnight	LLE (6M HCl+ n-butyl chloride: CAN:ethylacetate, 4:1:1)	LC–MS/MS, ESI+, MRM	YMC ODS-AQ column; MP: NA	[100]
Multiple drug groups								
AMP, MAMP, MDA, MDMA, MDEA, ketamine, norketamine, MTD, EDDP, MOR, COD, 6AM	10	0.08–5	N/A	Cut Methanol 25°C overnight	SPE	GC–MS, Full scan/SIM		[101]
AMP, MAMP, MDA, MDMA, COD, HYC, OXYC, HYM, OXYM, 6AM, MOR, COC, BE, CE, NORCOC, MTD, Meperidine	10 mg	0.5–8	2 × CH_2Cl_2	Cut 1 ml methanol 56°C 18 h	OASIS HCX cartridges HS-SPME	GC–MS, Full scan/SIM	Derivatization: BSTFA & MSTFA FactorFour column	[102]

6AM: 6-Acetylmorphine; AMP: Amphetamine; BE: Benzoylecgonine; BUP: Buprenorphine; BSTFA: N,O-bis(trimethylsilyl) trifluoroacetamide; CE: Cocaethylene; COC: Cocaine; COD: Codeine; EDDP: 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; ESI: Electrospray ionization; HS-SPME: Headspace-solid phase microextraction; HYC: Hydrocodone; HYM: Hydromorphone; LC–HRMS: Liquid chromatography-high resolution mass spectrometry; MAMP: Methamphetamine; MDA: 3,4-Methylenedioxyamphetamine; MDEA: 3,4-Methylenedioxyethylamphetamine; MDMA: 3,4-Methylenedioxymethamphetamine; MOR: Morphine; MRM: Multiple reaction monitoring; MSTFA: N-methyl-N-trimethylsilyltrifluoroacetamide; MTD: Methadone; NBUP: Norbuprenorphine; NORCOC: Norcocaine; OXYC: Oxycodone; OXYM: Oxymorphone; SIM: Single ion monitoring; SPE: Solid-phase extraction.

correlations between the analytical findings, the degree of *in utero* exposure and the clinical outcomes.

Future perspective

The future perspective in bioanalytical methods for the detection of *in utero* drug exposure is the development of confirmation and screening methods that include a broad spectrum of compounds, with high sensitivity, simple and fast. Among the new analytical techniques, HRMS looks the most promising. HRMS allows the detection of unlimited number of compounds and even identification of unknown ones. This technique will be useful in routine analyses as well as in research studies. With regard to sample preparation, the development of new strategies, that are faster than conventional methods and that use less amount of solvents, such as disposable pipette extraction tips or dilute and shoot, are interesting ways to explore.

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Executive summary

Background

- Drugs in pregnancy are a major public health concern.
- In USA, 15.9% pregnant women smoke, 8.5% drink alcohol and 5.9% report illicit drug use.

Effects of prenatal exposure to drugs of abuse, tobacco & alcohol

- *Pregnancy outcomes*
  - Miscarriage, preterm delivery, deep vein thrombosis, hypertension and placental abruption.
- *Short-term effects*
  - Neonatal abstinence syndrome (NAS), neurobehavioral or autonomic system disturbances, congenital anomalies and low birth weight.
- *Long-term effects*
  - Problems in neurodevelopment (memory, attention, language, learning skills and behavior).

Specimens for in utero drug exposure

- From mother: urine, blood/plasma, oral fluid, sweat and hair.
- From newborn: urine, meconium and hair.
- From maternal–fetal unit: placenta and umbilical cord.
- Maternal hair and meconium are the gold standards.
- Placenta and umbilical cord are promising alternative specimens.
- Hair, meconium, placenta and umbilical cord sample preparation involves sample pretreatment (hair wash and digestion, meconiun and tissue homogenization), sample extraction and instrumental analysis (GC-MS, LC–MS/MS).
- Analytical methods have to be fully validated.

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