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Detection and quantitation of non-steroidal anti-inflammatory drug use close to the time of birth using umbilical cord tissue

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ABSTRACT

Background: Nonsteroidal anti-inflammatory drugs are contraindicated in the third trimester of pregnancy due to negative effects including alteration of uteroplacental blood flow, premature ductus arteriosus closure, and adverse effects on the fetal kidney. However, many women are unaware of these risks, and commonly report their use in pregnancy. We aimed to determine if umbilical cord was a reliable matrix for detecting NSAID use, determine incidence of use close to labour, and uncover associations with obstetric/neonatal outcomes.

Methods: We developed a UHPLC-MS/MS method to simultaneously detect diclofenac, ibuprofen, indomethacin, naproxen, and salicylic acid in plasma and umbilical cord lysate. Using this method, we screened 380 lysates to determine the prevalence of NSAID use. Results were compared to the clinical outcomes in pregnancy using ICD9/10 chart codes (n = 21).

Results: The UHPLC-MS/MS method has excellent linearity, accuracy, and precision in solvent and plasma, but lower sensitivity in umbilical cord lysate. We report a 3 % rate of NSAID ingestion within days of labour – the pharmacokinetically-determined window for active ingestion. There were no significant differences observed for maternal, obstetric, or neonatal outcomes between the NSAID positive group (n = 11) and NSAID negative group (n = 369).

Conclusions: Because NSAID use in third trimester is contraindicated, even a 3% usage rate is alarmingly high. Based on UHPLC-MS/MS performance of umbilical cord lysate, 3% is likely a conservative estimate. Recent adoption of NSAIDs under clinical supervision to support *in vitro* fertilisation and prevent pre-eclampsia indicates future work should focus on determining safe dosages of NSAIDs and the correct therapeutic window in pregnancy.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are analgesics that inhibit cyclooxygenase (COX) 1 and 2 enzymes, thereby blocking the actions of prostaglandins and thromboxanes [1–3]. The NSAIDs are versatile and have many indications including general pain and fever, rheumatoid arthritis, and osteoarthritis [4]. As they have become increasingly available over-the-counter (OTC) NSAID use has increased in terms of number of people taking the drugs, amount (doses) of drug ingested, and expanded use in different pain-causing diseases and syndromes, within this context women more likely to be regular users than men [5,6].

The NSAIDs are contraindicated in the third trimester [7,8] due to altered utero-placental blood flow and increased incidence of oligohydramnios, persistent pulmonary hypertension of the newborn, and premature closure of the ductus arteriosus [9–12]. Indeed, indomethacin is an agent of choice for treating patent ductus arteriosus in neonates [13], demonstrating the risks of ingesting this drug close to birth.

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Furthermore, as prostaglandins play critical roles in the parturition process as well as renal function, NSAID ingestion in third trimester may adversely affect labour and delivery [14–20]. More recently, concern has been raised over NSAID use causing nephrotoxicity in the fetus [12,21–25]. Guidelines from the United States Food and Drug Administration specify that NSAIDs should be avoided in pregnancy in preference for acetaminophen [26].

Despite these risks being well-recognized by the medical community, most women are unaware that NSAIDs are contraindicated in the first and third trimesters of pregnancy. Additionally, because many NSAIDs are available OTC, there is a perception of safety in the general population. For women specifically, common conditions such as headache, joint pain, or menstrual pain are regularly treated with NSAIDs and so these patients often continue NSAID use into pregnancy [8].

Although NSAIDs are normally contraindicated in the third trimester, there are certain circumstances in which this class of drugs are used under medical supervision in the perinatal period. Specifically, this class of drugs is a common tocolytic and a treatment for patent ductus arteriosus [13,25]. With increasing use of this drug class being reported in pregnancy [7,8], there has been a higher incidence of adverse obstetric events [23,25,27]. As such, more information is needed regarding population use of this drug class in pregnancy, safe dosages, and safe timing of use in pregnancy. A screening method for NSAIDs in blood and reproductive tissues would benefit researchers and physicians in providing guidance on NSAID prescribing and OTC use in the perinatal period.

All NSAIDs cross the placenta to some degree [28–30], therefore, screening fetal tissues (e.g. placenta or umbilical cord (UC)) may be more informative than screening maternal blood, when fetal exposure is the risk measure. Presence of a drug in fetal tissues demonstrates that some level of exposure is occurring, but there are no currently defined dose:concentration relationships that would make this process predictive of maternal ingestion or absolute fetal exposure levels. With our prior experience using reproductive tissues for detection and quantification of drugs, endo- and xenobiotics and their effects on reproductive outcomes [31–36], we developed the hypothesis that UC would be a reliable matrix for detecting NSAID use, more reflective of fetal NSAID exposure than maternal blood alone. In this study, we retrospectively screened 380 UC lysates for five of the most commonly used NSAIDs: diclofenac, ibuprofen, indomethacin, naproxen, and salicylic acid, to determine maternal ingestion of these drugs close to birth, fetal exposure, and relationships (if any) between NSAID use and obstetric and neonatal outcomes.

2. Materials and methods

Aceclofenac, carprofen, diclofenac, ibuprofen, indomethacin, naproxen, and salicylic acid were from Sigma-Aldrich (Oakville, ON, Canada). HPLC-grade methanol and water were from Fisher Scientific (Ottawa, ON, Canada). All other reagents were from VWR International Ltd (Mississauga, ON, Canada) unless otherwise specified.

2.1. Human plasma collection

Whole blood from an anonymous donor free of NSAIDs for the previous 7 days was collected in sodium citrate tubes. Samples were centrifuged at 2000 × g for 20 min at 4 °C within 1 h of collection, then plasma aliquoted and frozen at −80 °C until use. The blood collection was performed with approval from The University of British Columbia's Research Ethics Board (H13-01805). Two commercial pooled human serum samples were also purchased for analysis from Bio-Rad Clinical Diagnostics (Irvine, CA, USA) and Innovative Research (Novi, MI, USA).

2.2. Human umbilical sample collection and processing

The human UCs used in this project (n = 380) were collected from Kapiolani Women's and Children's Hospital (Honolulu, HI, USA) with informed consent from the mother, including consent for future investigation. Inclusion criteria for the reproductive biorepository were women >18 years, no requirements for placenta to be sent to the pathologist, and live births only. Cords were collected within 8 h of birth, washed, and snap frozen in liquid nitrogen before archiving at −80 °C. Upon request, UCs were cut frozen, and transferred to The University of British Columbia on dry ice, never thawed. Tissues used in this study were collected between 2014/2015. This study was conducted under approval from the Clinical Research Ethics Board at the University of British Columbia (H14-00092) and The University of Hawaii IRB for Human Subjects (CHS 15080). Demographics of the cohort are presented in Table 1.

Umbilical cord tissue pieces were thawed at room temperature, wet-weight recorded, then mechanically homogenized 1:3 (w:v) in 100 mM Tris-HCl buffer containing 5 mM $MgCl_2$ (pH 7.4) and 2 mM phenylmethylsulfonylfluoride as previously described [35,37]. All samples were processed into lysates at the University of British Columbia in 2016 and archived at −80 °C for future studies.

2.3. UHPLC-MS/MS method development

Screening of diclofenac, ibuprofen, indomethacin, naproxen, and salicylic acid was performed with a novel UHPLC/MS/MS developed in-house as follows.

2.3.1. Instrumentation, chromatography and mass spectrometry conditions

The UHPLC/MS/MS system was an Agilent Infinity 1290 system (Agilent, Mississauga, ON, Canada) connected to an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer (AB Sciex, Concord, ON, Canada) and data acquired using the Analyst 1.5.2 software (AB Sciex).

The analytic column was a Acquity UPLC BEH C18, 1.7 µm, 2.1 × 50 mm, with an in-line C18 VanGuard (1.7 µm, 2.1 × 5 mm) guard column (Waters Corporation, Milford, MA, USA). Mobile phase A was 0.1 % formic acid in water and mobile phase B was 0.1 % formic acid in methanol with a flow rate of 0.3 mL/min, injection volume of 15 µL and run time of 10 min. Chromatographic conditions are reported in Table 2.

Diclofenac, ibuprofen, indomethacin, naproxen and salicylic acid were quantitated using multiple reaction monitoring with electrospray ionization in negative ion mode. The transitions used were: diclofenac

Table 1
Characteristics of the cohort.

Parameter	Clinical Characteristics of Pregnancies for Umbilical Cords Studied
Maternal Age (years, mean ± s.d.)	28.2 ± 6.3
Race	
Black	N = 6
White	N = 122
Asian	N = 141
Other	N = 111
Gestational Age (Weeks)	38.3 ± 2.3
Delivery Method	
Caesarian	15.8 %
Vaginal	84.2 %
Birth Weight (grams, mean ± s.d.)	3165 ± 609
Singleton	
Yes	98.4 %
No	1.6 %
Baby's Sex	
Male	54.7 %
Female	45.3 %
Drugs	
Cigarettes	N = 40
Alcohol	N = 0

Table 2
Gradient programming for chromatographic separation of analytes.

Time (min)	Flow Rate (mL/min)	Solvent A (%)	Solvent B (%)
0.0	0.3	85	15
5.0	0.3	2	98
7.0	0.3	2	98
7.1	0.3	85	15
10.0	0.3	85	15

m/z 294.0 → 250.0, *m/z* 294.0 → 214.0 (retention time 4.99 min); ibuprofen *m/z* 205.1 → 161.0 (retention time 5.10 min); indomethacin *m/z* 356.1 → 312.1, *m/z* 356.1 → 297.0, *m/z* 356.1 → 270 (retention time 4.99 min); naproxen *m/z* 229.0 → 185.0, *m/z* 229.0 → 170.1 (retention time 4.46 min); salicylic acid *m/z* 136.5 → 93.0 (retention time 3.37 min); aceclofenac *m/z* 352.0 → 75.0 (Fig. 1).

2.3.2. Preparation of Calibration Standards and Samples in solvent and human plasma

Stock solutions of NSAIDs were prepared in methanol at 1 mg/mL then working standard solutions diluted in water to 1−1000 ng/mL for standard curve calibration. The internal standard (IS, aceclofenac or carprofen) was dissolved in methanol at 1 μg/mL, then further diluted in water to working concentration (100 ng/mL).

Calibration standards in plasma were prepared on the day of analysis by pipetting 90 μL of plasma, 10 μL of standard and 40 μL of IS into glass tubes, blank plasma contained 10 μL HPLC grade water. An equal volume of 1 M hydrochloric acid was added to all samples, vortexed for 10 s and then 2 mL of methyl *tert*-butyl ether (MTBE) added. Mixtures were vortexed for 30 s, frozen at −80 °C for 15 min, removed from −80 °C and the organic layer transferred to a clean tube. The organic layer was evaporated under nitrogen at 35 °C, and residues reconstituted in methanol/HPLC grade water 50:50 (v/v, 100 μL).

2.4. Method validation

The UHPLC-MS/MS method was validated for use in human plasma and umbilical cord lysate according to the Guidelines for Bioanalytical Method Validation published by the United States Food and Drug Administration [38]. Linearity, limits of sensitivity, accuracy, precision, matrix effects, and recovery were determined. Additional stability studies were performed for plasma.

2.4.1. Linearity and limits of sensitivity

Standard curves were generated over the linear range of each analyte, where the coefficient of variation (CV) was <15 % of each concentration, except the lower limit of quantitation where CV was ≤20 %. The limit of sensitivity was determined by calculating 3X signal-to-noise ratio. Linearity was determined by a linear regression, using $1/x^2$ least squares weighting, with a minimum acceptable linearity of $r^2 = 0.985$. If an analyte was below the lower limit of quantitation but above the limit

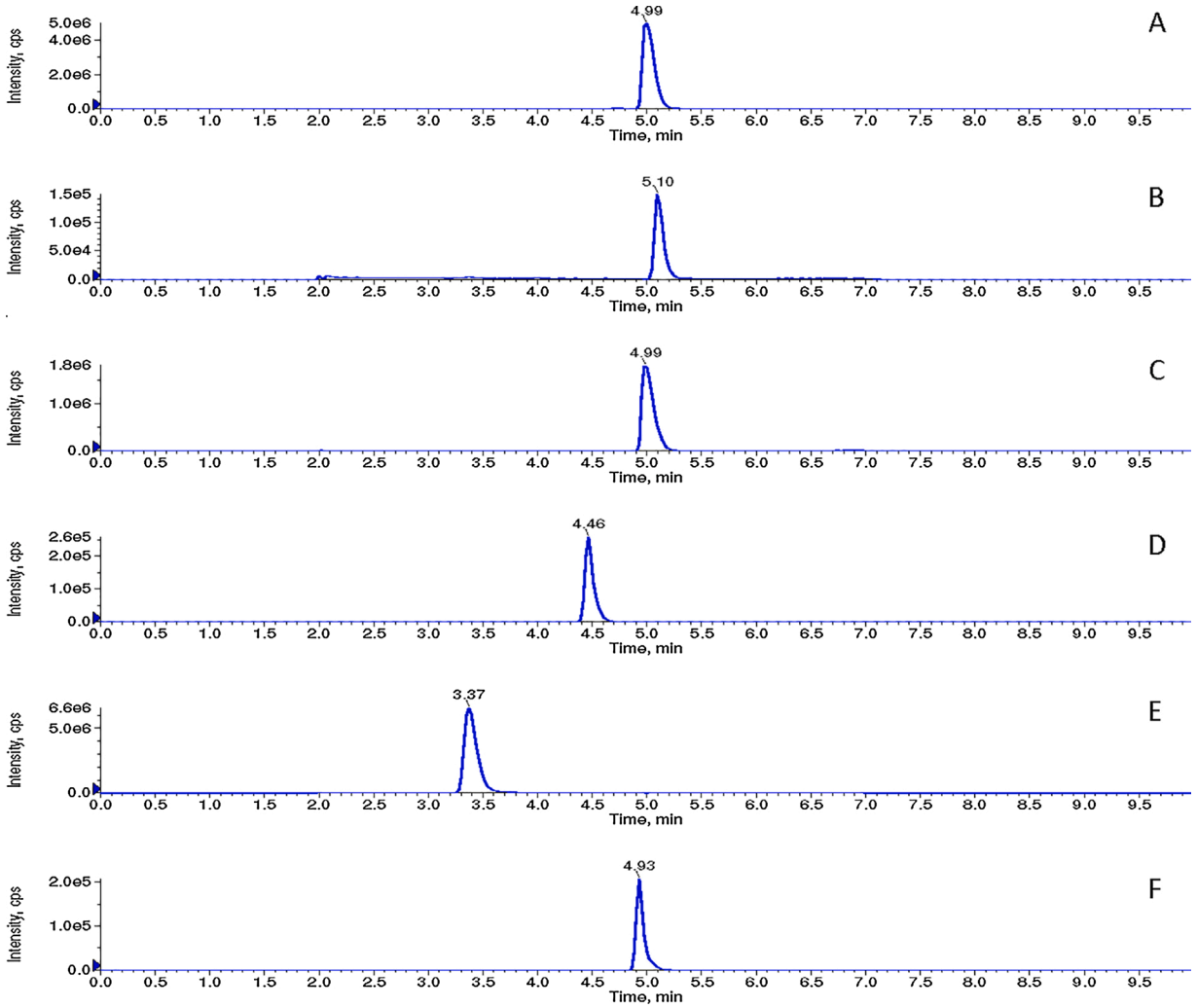

Fig. 1. Chromatograms of NSAIDs analyzed in multiple reaction monitoring modes. Calibration standard at 500 ng/mL. **A.** Diclofenac (294.0/250.0) **B.** Ibuprofen (205.1/161.0) **C.** Indomethacin (356.1/312.1) **D.** Naproxen (229.0/185.0) **E.** Salicylic acid (136.5/93.0) **F.** Aceclofenac (352.0/75.0).

of sensitivity it was assigned "present" but not quantitated.

2.4.2. Accuracy and precision

Intra-assay and inter-assay accuracy and precision were determined at three quality control (QC) concentrations designated: QC_{low} at 35 ng/mL, QC_{mid} at 200 ng/mL, and QC_{high} at 400 or 800 ng/mL, depending on the linear range of the analyte. Intra-assay accuracy is calculated as a percentage of the nominal concentration for each QC, evaluated in triplicate. Intra-assay precision (CV) was also evaluated in triplicate. Inter-assay accuracy and precision were evaluated over a minimum of three different validation batches.

2.4.3. Recovery and matrix effects

Recovery was determined in plasma or UC lysate by comparing the peak area ratios of samples spiked prior to extraction, with samples spiked after extraction. Matrix effects of human plasma or UC lysate on the analytes were assessed by comparing mean peak areas of analytes in extracted plasma, to mean peak areas of standard solutions spiked in solvent. Matrix effects were determined at three concentrations (QC_{low}, QC_{mid}, QC_{high}) each in triplicate. The IS normalized matrix effect was calculated by the matrix effect of each analyte divided by the matrix effect of the IS.

2.4.4. Stability

The stability of each NSAID in plasma was evaluated at the QC concentrations when placed on the benchtop at 20 °C for 24 h reflecting standard laboratory temperature in Canada and maximum time between blood draw and transfer to the laboratory (this never exceeded 2 h in our study but may do so in clinical laboratories). To test freeze-thaw effects, QCs were also spiked into blank plasma and frozen at −80 °C for 24 h then thawed to room temperature and analyzed. Finally, samples were commonly queued in the plate injector at 4 °C for up to 24 hs, hence standard curves were routinely analyzed at the end of the 24 h injection period to determine lability over time at the temperature.

2.5. NSAID screening in UC lysate by UHPLC-MS/MS

2.5.1. Preparation of calibration standards and samples in UC lysates

Calibration standards in blank UC lysate were prepared as described above except with 90 μL of UC lysate rather than plasma. Due to instability of aceclofenac in UC lysate, the IS was changed to carprofen (200 ng/mL). Carprofen was monitored at *m/z* 272.0 → 226.0, *m/z* 272.0 → 228.0.

2.6. Pharmacokinetic analysis

Pharmacokinetic analyses were performed to determine predicted maximum concentrations (Cmax) of NSAIDs in maternal blood after a single oral dose and the window of detection before effective biological elimination (using five half-lives post ingestion as the biological elimination parameter). This gave drug levels representative of active ingestion of single oral doses, both at the lowest effective dose, and highest recommended for analgesia for each NSAID and, particularly for salicylic acid that can be ingested in the diet; allowed us to exclude incidental drug levels. Due to lack of information on transfer, accumulation, and metabolism of NSAIDs within the fetal compartment, a pharmacokinetic analysis cannot be performed for the expected concentration in the umbilical cord. Therefore, levels of NSAIDs in maternal blood after dosing do not necessarily correspond to concentration in the UC.

3. Results

3.1. Optimization of chromatography and mass spectrometry conditions

After repeated trials of varying LC–MS/MS conditions, the conditions presented here were chosen, as they provided narrow and symmetrical peaks with the shortest run time of 10 min. Mobile phase B (methanol) give higher abundance for compounds with late retention times as compared to results when acetonitrile was used as mobile phase B. Additionally, 0.1 % v/v formic acid was added to both mobile phases because it gave sharper peaks for compounds with earlier retention times compared to 2.5 mM ammonium formate. During method development, calibration curves were constructed after extraction at acidic, basic and neutral pH. All NSAIDs extracted better at an acidic pH, hence 100 μL of 1 M HCl was added to each sample prior to liquid-liquid extraction. The *m/z* transitions used for quantification of each compound were chosen based on the abundance of their daughter ions. A flow rate of 300 μL/min resulted in better peak shape than other flow rates that were tested.

3.2. Method validation

3.2.1. Linearity and limits of sensitivity in solvent, plasma and UC lysate

The calibration curves were evaluated a minimum of three times in solvent, plasma, and UC lysate. The limit of sensitivity was below 1 ng/mL for all analytes and linearity above 0.99 for all analytes (Table S1).

3.2.2. Accuracy and precision in plasma and UC lysate

The accuracy and precision of the plasma and UC lysate curves are presented in Table S2. Intra-day and inter-day accuracy and precision were within ±15 % of the nominal concentration, and CVs were <15 %.

3.2.3. Stability, matrix effects and recovery in plasma and UC lysate

Stability studies (Table S3) showed that all analytes were stable at room temperature for 24 h. Analytes were stable at −80 °C for one freeze-thaw cycle in plasma with the exception of ibuprofen and salicylic acid, where the concentrations of ibuprofen and salicylic acid QC_{low} and QC_{mid} showed deviation up to 40 % and 30 %, respectively (Table S3). Hence freezing and thawing ibuprofen and salicylic acid in plasma, even once, is associated with significant degradation and/or loss to extraction. Samples in solvent frozen at −80 °C for up to 6 months were not different to freshly prepared samples and the single thaw did not cause loss of analyte. Finally, samples were stable in the UHPLC auto-sampler with acceptable accuracy, precision, resolution time and peak shape up to 24 h, indicating that samples can be queued and analyzed with no loss of stability at 4 °C for up to one day (*data not shown)*.

The matrix effect of human plasma and UC lysate on the analytes is presented in Table S4. The matrices affected accuracy differently at different concentrations, for this reason it is recommended that calibration curves be prepared in blank matrix rather than solvent (as we did).

Recovery in human plasma and UC lysate is presented in Table S5. Most compounds showed good values for recovery; however, the recovery of ibuprofen was reduced at QC concentrations of 35 and 200 ng/mL. The IS aceclofenac had a low recovery in plasma, leading to a larger area ratio and therefore a recovery in excess of 100 % for indomethacin, naproxen, and salicylic acid.

3.3. Detection of NSAIDs in human plasma

The individual donor was negative for all NSAIDs. Pooled human plasma from Bio-Rad (Irvine, CA, USA) was positive for diclofenac and indomethacin but detection was below the lower limit of quantitation, while ibuprofen (254 ng/mL) and naproxen (1320 ng/mL) and salicylic acid (387 ng/mL) were quantifiable. In contrast, the pooled sample from Innovative Research (Novi, MI, USA) diclofenac and indomethacin were not detected, but ibuprofen (897 ng/mL), naproxen (1670 ng/mL) and salicylic acid (844 ng/mL) were quantified. Differential detection and quantitation of drugs were reported in respective pooled samples, showing method selectivity and sensitivity.

3.4. Detection of NSAID use by pregnant women using UC Lysate

To reliably use UC lysate for NSAID screening, the IS had to be replaced. The original IS (aceclofenac) validated in plasma was unstable in UC lysate. Over the course of standard curve and QC injection (approximately 5 h), the internal standard count would drop to 55 % of starting values. To ensure that increasing concentration of other NSAIDs in the method were not interfering with aceclofenac ionization, we injected the same sample over a 5 -h time period and the same decline was observed. Phenylbutazone, another NSAID which is only used in veterinary medicine, was tested to use as the IS. Over the course of an 8-h run time period, phenylbutazone counts also declined to 45 % of starting values, thus it too was judged unsuitable/unstable. Finally, carprofen was confirmed as a stable and suitable IS for use in UC lysate. Over the 8 -h test of standard curve and QC injection, area counts of phenylbutazone were within ±15 % of the starting values. Screening batches of samples were routinely queued over 16 h, and carprofen was stable for the duration of this time.

In total, 380 UC lysates were screened with two samples positive for ibuprofen, one sample positive for indomethacin, and eight samples positive for salicylic acid. For ibuprofen and indomethacin, the positive samples were below the limit of quantitation, but above the limit of sensitivity of the assay. These samples are classified as “positive” but cannot be quantified. For salicylic acid, the eight concentrations detected were 27.0, 42.3, 45.4, 48.1, 81.0, 99.1, 108.0, and 423.0 ng/mL. Because time-since-ingestion is unknown, we cannot back-calculate

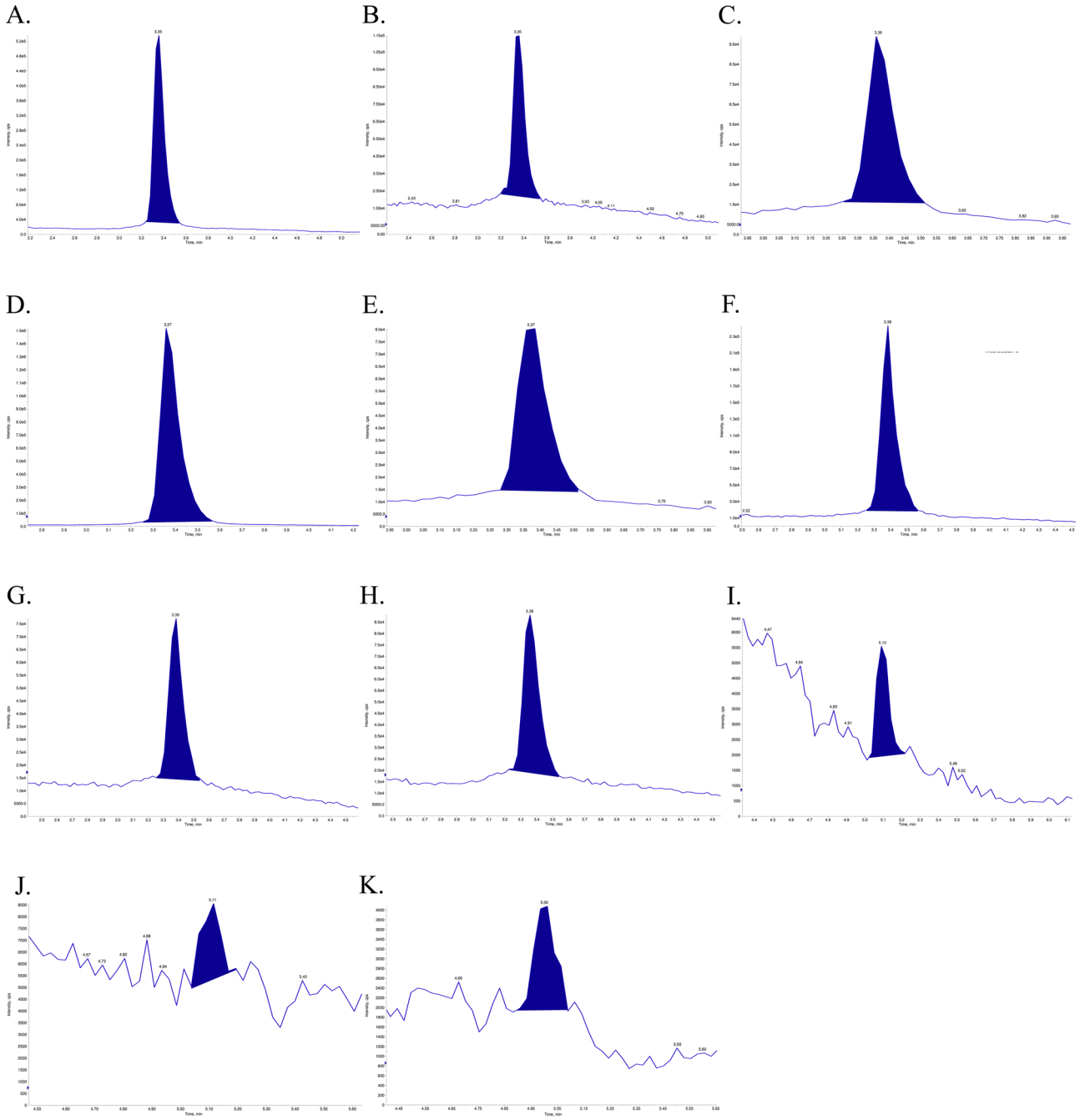

Fig. 2. Chromatograms of UC samples positive for NSAIDs. **A.** Sample 555, SA 99.1 ng/mL **B.** Sample 464, SA 27 ng/mL **C.** Sample 186, SA 48.1 ng/mL **D.** Sample 232, SA 432 ng/mL **E.** Sample 491, SA 42.3 ng/mL **F.** Sample 541, SA 45.4 ng/mL **G.** Sample 481, SA 108.0 ng/mL **H.** Sample 545, SA 81.0 ng/mL **I.** Sample 217, IBU below LLOQ **J.** Sample 508, IBU below LLOQ **K.** Sample 250, IND below LLOQ.

individual doses. Overall, 2.9 % of samples tested positive for an NSAID, with the most common being salicylic acid. Traces of all eleven positive samples are presented in Fig. 2.

No significant differences between NSAID positive samples and negative samples were observed for maternal age, maternal BMI, gestational age, gestational diabetes mellitus, membrane rupture, premature rupture of the membranes, preterm labour, baby weight, and baby sex. There were no cases of NICU admission, IUGR, gestational hypertension, preeclampsia, HIV, cancer, or cardiac disease in mothers who were NSAID positive. A subset of UC samples were from women under care at a clinic for pregnant women with addiction and dependency issues. These women were regularly screened for phencyclidine, benzodiazepines, cocaine, amphetamine, opiates, barbiturates, and alcohol in a hospital laboratory. None of the NSAID positive mothers had positive screens for these drugs. All were vaginal delivery, and labour proceeded normally and spontaneously.

3.5. Pharmacokinetic analysis

For a single oral dose, after 5 half-lives (effective biological elimination of the dose), the concentration of NSAIDs expected in the blood would range 4.3–556 ng/mL for the lowest effective analgesic dose recommended, and 11.1–1361 ng/mL after the highest dose recommended (Table 3). These are the concentrations above which NSAIDs needed to be detected to make a determination of "active recent ingestion". The limits of sensitivity for the UHPLC/MS assay are far below this window, meaning we have reliable detection of active ingestion. The exception to this statement is salicylic acid, which can be ingested from dietary sources [39]. Because of method sensitivity, it is possible that false positives would occur at concentrations equivalent to 2.47 μmol/L and below in plasma. This concentration is the maximum feasible level acquired from dietary ingestion in the Western diet [39,40]. All samples reported as "positive" for salicylic acid were above the lower limit of quantification of the UHPLC-MS/MS assay and above the pharmacokinetically-determined active drug ingestion threshold.

4. Discussion

Here we report that 3 % of women used an NSAID close to birth, with two ingesting ibuprofen and one ingesting indomethacin, whilst eight women used aspirin, likely within days of labor and delivery. Because NSAIDs are contraindicated in the third trimester, even a 3% usage rate in the days adjacent to birth is alarmingly high. Our reported rate is similar to a previous study where self-reported NSAID use was ~5 % [8] and implies that these data are reliable, if conservative. Associations between *in utero* NSAID exposure and nephrotoxicity in neonates have been reported [25,23], with increasing severity of outcome as NSAIDs were ingested closer to birth [23,27]. Additionally, NSAID exposure has been associated with increased risk of major malformations and spontaneous abortion (Nakhai-Pour and Berard, 2008; Daniel et al., 2014). Because no dose:response relationships have been determined in any of these studies despite proposed mechanisms through e.g. altered prostaglandin signalling, there is uncertainty around the timing and/or concentrations of NSAID exposure that might adversely affect pregnancies.

Table 3
Pharmacokinetic analysis of NSAID levels in plasma following 5 half-lives for low and high dose regimens.

Drug	Time to five half-lives (days)	Low dose concentration in blood after five half-lives (ng/mL)	High dose concentration in blood after five half-lives (ng/mL)
Salicylic Acid	4.2	409.9	1157.7
Diclofenac	0.4	4.3	11.1
Ibuprofen	0.4	156.0	1361.7
Indomethacin	0.9	9.8	19.9
Naproxen	3.5	556.0	842.4

In performing this screening study, we successfully created and validated a novel UHPLC-MS/MS method and used it to simultaneously detect and quantify diclofenac, ibuprofen, indomethacin, naproxen, and salicylic acid in human plasma and UC lysate. This is the first UHPLC-MS/MS method (to our knowledge), to do so in a single analytical pass in biological matrices, and certainly the first to be applied to umbilical cords. Salicylic acid was included in the method, and not acetylsalicylic acid (aspirin), because the parent drug is unstable in most organic solvents and is also hydrolyzed in blood to salicylic acid within 15 min of ingestion [41].

Some technical limitations of this study are relevant to the interpretation of our findings. Although our analytical method can simultaneously detect and quantitate these five NSAIDs, the biological matrices presented some challenges for analysis. During development, we noted that plasma and UC lysate exhibit matrix effects, therefore NSAID calibration curves should be prepared in a blank sample of the same matrix used for screening (as we did). Also, the lower sensitivity observed in UC as compared to blood plasma also means that our results likely underestimate both concentrations and prevalence of NSAID use. A second potential limitation of this study is also the use of archival samples. The whole UC had been stored for 1–12 months at −80 °C before shipping and processing to lysates, and lysates were stored for up to 3 years before screening, because these UCs have been used in multiple studies. Our stability study indicates that all analytes were stable in solvent at −80 °C for up to 6 months, but beyond that we cannot rule out analyte degradation during storage. Although this introduces some uncertainty into our absolute values, these stability concerns underscore the conservative nature of our findings, implying that the prevalence of actual NSAID ingestion may be even higher. Lastly, the way in which drugs and other compounds distribute into the UC and subsequently the UC-specific half-life (which confers the viable window for detecting drugs), is not known for NSAIDs for to our knowledge, for any other drug or chemical (Wright, 2015). As the UC is mostly comprised of glycosaminoglycans [42] the expectation, based on physicochemical considerations, is that hydrophilic NSAIDs would show affinity for the UC, however this statement is empirical and limited by lack of actual experimental results. Previous studies that have attempted to predict the concentration of NSAIDs in the fetal compartment discuss the importance of determining parent NSAID levels, as opposed to the metabolites [22,29]. Certain NSAIDs, including indomethacin, readily cross the placenta and fetal levels can be expected to be similar to maternal levels [22]. Additionally, due to low expression levels and activities of drug metabolizing enzymes in the fetal liver the level of parent drug is not expected to decline rapidly due to metabolism. Concentration of salicylic acid in the fetal compartment can be much higher than that of the maternal blood [29].

In addition, there are some clinical limitations to our study. We only received a small number of ICD9/ICD10 chart codes, and our data lacked any information on chart notations, so drugs prescribed in pregnancy or during labour and birth were not captured in our data. A specific example of medical use of NSAIDs during pregnancy is prescribing aspirin to women with a high risk of preeclampsia, which has become standard clinical practice in the last few years [43,44]. The UCs used in our study were collected in 2014/2015 before this indication became common, so this specific factor is unlikely to be the source of aspirin reported. However, this highlights that medical practice is constantly changing based on clinical experience and patient need, and where a drug or class of drugs such as NSAIDs were previously contraindicated (and remain so "officially"), as clinical practice changes, the pharmacology and toxicology of all drugs should continuously be re-evaluated in order to manage risk.

Based on the results of our screening study and expected concentrations calculated in the pharmacokinetic analysis, some improvements could be made to improve the sensitivity of the method, particularly for

ibuprofen, diclofenac, and indomethacin. If the method is adapted for use in matrices which exhibit a matrix effect, solid phase extraction would likely result in a cleaner sample and aid in mitigating the matrix effect. Additionally, the use of deuterated internal standards for each compound would increase sensitivity by accounting for recovery, differences in ionization, and retention time. Using these strategies to further optimize the method would be beneficial for population NSAID screening, when the time since last dose may be unknown or expected concentrations are on the lower end of the curve.

Here, a moderate cohort of n = 380 is presented – more than many researchers have access to – yet was still hampered by low numbers of "positive" drug ingestions/exposures. This underlines the need for screening large numbers of samples when low rates of prevalence occur for any potential risk factor or outcome. Hence, our report that NSAID use close to birth was not associated with clinical outcomes requires confirmation in a much larger cohort. On the other hand, this also highlights the value of biorepositories that can supply large sample-sets on request, saving the time and expense of months or years of prospective collection.

A prevalence of 3% NSAID ingestion immediately adjacent to birth is concerning on an epidemiologic level, even more so because given that our findings are conservative. Although there are known risks of *in utero* NSAID exposure for fetal development, partition, and neonatal health [10–12], the re-emergence of these drugs – both self-administered and under physician care – may precipitate unexpected outcomes. More public education is needed so that pregnant women understand the risks of choosing to ingest NSAIDs, and do so only under appropriate circumstances, or not at all. Despite that there is not currently an acute public health crisis related to NSAID use in pregnancy, recent studies clearly demonstrate increasing medical and OTC use of NSAIDs [7,8] and concurrently higher population incidence of adverse obstetric events [23,25,27]. Therefore, there is a need to determine safe dosages and timing of administration for NSAIDs in pregnancy to support best-practice in medical care.

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CRediT authorship contribution statement

Hayley R. Price: Data curation, Formal analysis, Methodology, Validation, Writing - original draft, Writing - review & editing. **Dickson Lai:** Methodology, Validation, Writing - review & editing. **Hugh Kim:** Funding acquisition, Writing - review & editing. **Tricia E. Wright:** Conceptualization, Funding acquisition, Writing - review & editing. **Michael W.H. Coughtrie:** Supervision, Formal analysis, Writing - review & editing. **Abby C. Collier:** Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

Dr. Collier declares that she has been a paid consultant for Genentech within the last 5 years. None of that work is related to this research project. The remaining authors report no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.09.003.

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