LC-MS/MS METHOD FOR THE DETERMINATION OF ANTIDEPRESSANTS AND BENZODIAZEPINES IN MECONIUM

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Abstract

An LC–MS/MS method for the determination of 14 benzodiazepines (alprazolam, α-hydroxyalprazolam, clonazepam, bromazepam, diazepam, nordiazepam, lorazepam, lormetazepam, oxazepam, flunitrazepam, 7-aminoflunitrazepam, triazolam, midazolam and zolpidem) and 15 antidepressants (amitriptyline, nortriptyline, imipramine, desipramine, clomipramine, norclomipramine, fluoxetine, norfluoxetine, sertraline, norsertraline, paroxetine, venlafaxine, desmethylvenlafaxine, citalopram and desmethylcitalopram) in meconium was developed and validated. Meconium samples (0.25±0.02g) were homogenized in methanol and subjected to mixed-mode cation exchange solid-phase extraction. Chromatographic separation was performed in reversed-phase, with a gradient of 0.1% formic acid in 2 mM ammonium formate and acetonitrile. Two different chromatographic gradient methods were employed, one for the separation of antidepressants and another for benzodiazepines. Analytes were monitored by tandem mass spectrometry employing ESI+ in MRM mode (2 transitions per compound). Method validation included: linearity (n=5, LOQ to 400 ng/g), limits of detection (n=6, 1-20 ng/g), limits of quantification (n=9, 5-20 ng/g), selectivity (no endogenous or exogenous interferences), accuracy (n=15, 90.6-111.5%), imprecision (n=15, 0-14.6%), matrix effect (n=10, -73% to 194.9%), extraction efficiency (n=6, 35.9-91.2%), process efficiency (n=6, 20.1-188.2%), stability 72 h in the autosampler (n=3, -8.5% to 9%) and freeze/thaw stability (n=3, -1.2 to -47%). The method was applied to 4 meconium specimens, which were analysed with and without hydrolysis (enzymatic and alkaline). The authentic meconium samples tested positive for alprazolam, α-hydroxyalprazolam, clonazepam, diazepam, nordiazepam, fluoxetine, norfluoxetine, clomipramine and norclomipramine. Therefore, the present LC-MS/MS method allows a high throughput determination of the most common benzodiazepines and antidepressants in meconium, which could be useful in clinical and forensic settings.

Keywords

Antidepressant, benzodiazepine, pregnancy, meconium, in utero exposure, LC-MS/MS

Introduction

Depression and anxiety disorders have the highest prevalence in women during the childbearing years (1). It is estimated that 12.4% of pregnant women suffer from depression (2), while anxiety disorders are present in 15.2% of them (3). In addition, anxiety disorders are the most prevalent concurrent disorders in depressed pregnant women (42.7%) (2). The main drugs used in the treatment of these diseases are benzodiazepines and antidepressants. Between 2006 and 2011, 6.5% of pregnant women in the US were exposed to antidepressants during pregnancy (4); moreover, a similar prevalence was found in a more recent study from 2016, in which 6 % of the women were exposed to the most commonly used antidepressants (selective serotonin reuptake inhibitors) during pregnancy (5). In relation to benzodiazepines consumption in pregnant women, a prevalence of 3.9% was found between 2006 and 2011 in the US (4). Specifically, a recent study carried out in our region (Galicia, Spain) found out that depression and anxiety disorders treatment decreased during pregnancy (3.4% six months before pregnancy vs 1.3% during pregnancy for depression, and 3.6% six months before pregnancy vs 1.8% during pregnancy for anxiety disorders) (6). This may be due to the fact that most of these drugs are listed in cathegory C or D by the FDA, which indicates that risk of using these drugs during pregnancy cannot be ruled out.

Depression and anxiety disorders during the perinatal period can negatively influence the health and well-being of the mother and the newborn, causing placental abruption, miscarriage, premature delivery, low birth weight, perinatal complications, and possible long-term childhood behavioral problems (1). However, treatment of these disorders with antidepressants and benzodiazepines can also produce serious obstetric complications, in fetal development, as well as during later life. Some of the problems associated with the use of these drugs during pregnancy are low birth weight, low Apgar score, spontaneous

abortions, premature births, increased risk of respiratory distress, discontinuation syndrome or increased risk of cardiac septal defects (7-12). Therefore, the treatment of women with psychiatric disorders during pregnancy is a challenge, and risks-benefits balance should be carefully evaluated (1).

Maternal interview has usually been employed to identify drug use during pregnancy; however, drug consumption is usually underestimated due to maternal underreport. A more objective procedure for identification of in utero drug exposure is the analysis of maternal and/or fetal biological matrices. For this purpose, several methods have been developed for the determination of antidepressants and/or benzodiazepines in different matrices, including maternal hair (13-18), meconium (16,17,19-22), neonatal hair (20,21,23,24), neonatal urine (21), umbilical cord blood (25,26) or placenta (21). Each matrix has advantages and disadvantages, depending on the ease of sample collection, the detection time window and the detection limit (27-29). Meconium, the first fecal matter of the neonate, is considered the reference matrix for the identification of drug use during pregnancy, since its analysis allows the detection of direct fetal exposure to drugs consumed in the second and, mainly, third trimester of pregnancy. Its formation begins between the 12^{th} and 16^{th} week of gestation and its expulsion takes place a couple of days after birth, with the advantage of an easy and non-invasive collection. In meconium both parent drugs and metabolites are present in concentrations of ng/g and, generally, drug concentrations remain stable when stored at <-20°C. Nevertheless, the main disadvantage of meconium is that it may not be available in cases of fetal distress, when discharge occurs before delivery (27-29).

The objective of the present work was to develop and validate a comprehensive LC-MS/MS method for the simultaneous determination of the most often prescribed

antidepressants and benzodiazepines for pregnant women, including some of their major metabolites.

Materials and methods

Chemicals

Amitriptyline, nortriptyline, imipramine, desipramine, clomipramine, norclomipramine, fluoxetine, norfluoxetine, sertraline, paroxetine, venlafaxine, desmethylvenlafaxine, citalopram, desmethylcitalopram, alprazolam, α-hydroxyalprazolam (α-OH-alprazolam), clonazepam, bromazepam, diazepam, nordiazepam, lorazepam, lormetazepam, oxazepam, flunitrazepam, 7-aminoflunitrazepam, triazolam, midazolam and zolpidem standards at 1 mg/mL in methanol, norsertraline at 100 µg/mL in methanol, and the internal standards (IStd) venlafaxine-d_6, sertraline-d_3, citalopram-d_6, imipramine-d_3, paroxetine-d_6, fluoxetine-d_6, nortriptyline-d_3, clomipramine-d_3, norfluoxetine-d_6, desipramine-d_3, alprazolam-d_5, clonazepam-d_4, oxazepam-d_5, diazepam-d_5, nordiazepam-d_5, zolpidem-d_6, lorazepam-d_4, flunitrazepam-d_7, 7-aminoflunitrazepam-d_7, α-OH-alprazolam-d_5 at 100 µg/mL in methanol were obtained from Cerilliant (Round Rock, TX, USA). Dichloromethane, ammonium formate, formic acid and acetonitrile were supplied by Scharlau (Sentmenat, Catalonia, Spain), purified water and ammonium hydroxide by VWR (Radnor, Pennsylvania, USA), and 2-propanol and methanol by Fisher Chemicals (Loughborough, Leicestershire, UK). Oasis MCX cartridges (3 cc, 60 mg) were from Waters Corp. (Milford, MA, USA).

Meconium samples

For the preparation of the calibration curves and quality control (QC) samples, we employed anonymized blank meconium specimens supplied by the University Hospital of Vigo (Spain). Blank meconium specimens were from newborns whose mothers

reported no benzodiazepines or antidepressants use during pregnancy. Moreover, to probe the applicability of the method and the hydrolysis step evaluation, 4 specimens from women on antidepressants or benzodiazepines treatment were collected. Real samples collection was approved by the Ethics Committee of the University of Santiago de Compostela (Spain) and by the Galician Clinical Research Ethics Committee (Xunta de Galicia, Spain; code number: 2011/203), and procedures were in accordance with the tenets of the Helsinki Declaration.

Preparation of calibration and QC solutions

A mixed standard solution at 10 µg/mL was initially prepared in methanol from the individual commercial ampoules. Further working solutions were prepared by dilution at 1, 0.5, 0.1 and 0.05 µg/mL. Calibration curves were prepared with 7 concentration levels (5, 10, 20, 50, 100, 200, and 400 ng/g) by addition of 25, 50 or 100 µL of the appropriate working solution to meconium blank samples.

Using a different 10 µg/mL initial solution, working solutions at 0.05, 0.1 and 0.5 µg/mL in methanol were used to elaborate low, medium and high QC samples (15 ng/g, 30 ng/g and 150 ng/g, respectively). For norsertraline, desmethylvenlafaxine and lorazepam only low (30 ng/g) and high (150 ng/g) QC samples were prepared.

A working solution containing the IStd (venlafaxine-d_6, sertraline-d_3, citalopram-d_6, imipramine-d_3, paroxetine-d_6, fluoxetine-d_6, nortriptyline-d_3, clomipramine-d_3, norfluoxetine-d_6, desipramine-d_3, alprazolam-d_5, clonazepam-d_4, oxazepam-d_5, diazepam-d_5, nordiazepam-d_5, zolpidem-d_6, lorazepam-d_4, flunitrazepam-d_7, 7-aminoflunitrazepam-d_7, α-OH-alprazolam-d_5) was prepared in methanol at a concentration of 1 µg/mL.

Meconium analysis

We weighted 0.25±0.02 g of meconium into Pyrex® tubes, which were homogenized with 2 mL of methanol, after adding 25 µL of the IStd at 1 µg/mL. After sonication for 30 min, the sample was centrifuged for 10 min at 4000 rpm. The supernatant was evaporated to dryness under nitrogen at 35°C, and the extract was reconstituted in 200 µL of MeOH, vortex-mixed, and 2 mL of 2% formic acid in water was subsequently added before performing the solid phase extraction (SPE) procedure.

For the SPE, samples were extracted with mixed mode reversed phase-cation exchange cartridges (Oasis MCX). Supernatants were directly loaded, and cartridges were washed afterwards with 2 mL of 2% formic acid in water and 2 mL methanol:water (50:50, v/v). After drying under vacuum for 10 min, analytes were eluted with 3 mL dichloromethane:2-propanol:ammonium hydroxide (75:24.5:0.5, v/v/v). The eluates were evaporated to dryness under nitrogen at 35°C, and reconstituted in 100 µL of 0.1% formic acid in 2 mM ammonium formate and acetonitrile (70:30, v/v).

Hydrolysis assessment

We tested basic and enzymatic hydrolysis to evaluate the necessity of breaking down possible phase II metabolites in order to detect the unbounded analyte. For the basic hydrolysis the protocol previously published by Lendoiro et al. was followed (30): meconium was sonicated with 2 mL of methanol for 30 min, and then centrifuged for 10 min at 4000 rpm; the sample was hydrolyzed with 25 µL of 12 M KOH at 60°C for 30 minutes and neutralized afterwards with 300 µL of 1 M HCl; finally, the supernatant was evaporated and reconstituted to perform the SPE procedure as described in section 2.4. Enzymatic hydrolysis was carried out following the protocol described by Marin et al. (22): methanol used for sample homogenization was evaporated to <1 mL, and 2 mL of β-glucuronidase enzyme (5000 units/mL in 0.1 M sodium acetate buffer, pH 5) were then added; after incubating at 60°C for 2 h, the supernatant was evaporated and subsequently

reconstituted to perform the SPE as previously described. To test the hydrolysis effect, 4 real positive samples were analyzed without hydrolysis and results compared to those achieved when basic or enzymatic hydrolysis were performed.

LC-MS/MS Instrumentation

An Alliance 2795 Separation Module with an Alliance series column heater/cooler (Waters Corp., Milford, USA) was employed. An X-Bridge Shield RP18 (100 mm x 2.1 mm, 3 μm) analytical column (Waters Corp.) was used for chromatographic separation at 35°C. The mobile phase was formic acid 0.1% in 2 mM ammonium formate (A) and acetonitrile (B) at a flow rate of 0.3 mL/min, although two different chromatographic gradient methods were employed, one for the separation of antidepressants and another for benzodiazepines. Therefore, each sample was injected twice for the analysis of all the compounds. For antidepressants, solvent B was 30% for the first 2 minutes, and linearly increased to 33% in 3 min, and then to 40% in 1.5 min, to finally to return to initial conditions at minute 7, with a total run time of 10 min. For benzodiazepines, solvent B linearly increased from 30% to 33% in 5 minutes, to 35% in 1.5 min, to 50% in 2.5 min, and then to 90% in 0.5 min and maintained for another half minute. Finally returned to initial conditions at minute 10.5, with a total run time of 15 min. The autosampler was maintained at 6°C.

A Quattro Micro™ API ESCI triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) was used for analyses. The instrument was operated in electrospray positive mode (ESI+) with the following optimized settings: capillary voltage 0.5 kV; source block temperature 150°C, desolvation gas temperature 400 °C, desolvation gas flow rate 800 L/h and cone gas flow rate 60 L/h. Data were recorded on multiple reaction monitoring (MRM) mode. MassLynx 4.0 software was employed to control data acquisition and QuanLynx 4.1 for data-processing (Waters Corp.).

Method validation

Method validation was performed according to the Scientific Working Group for Forensic Toxicology (SWGTOX) standard practices for method validation in forensic toxicology (31). The following validation parameters were assessed: selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, imprecision, matrix effect, extraction and process efficiency, autosampler stability and freeze/thaw stability. The protocol for the validation of each parameter and acceptance criteria are shown in in Table S1 (Supplementary Data).

Application to real specimens

To confirm method applicability, four authentic meconium specimens from pregnant women on antidepressants and/or benzodiazepines treatment during pregnancy (fluoxetine, clomipramine, clonazepam, alprazolam and/or diazepam) were analyzed using the described LC-MS/MS method.

Results

Method development and validation

Chromatographic separation of all the antidepressants and their metabolites was achieved in 4 minutes, with a total run time of 10 minutes; whereas, benzodiazepines chromatographic separation was completed in 9 minutes, with a total run time of 15 minutes. Two MRM transitions were monitored for each analyte, and the most intense transition was used for quantification. MRM transitions, cone voltage (CV), collision energy (CE) and retention time (Rt) for the compounds and the deuterated analogues are shown in Table 1.

Endogenous and exogenous selectivity was verified, since no interferences were found at the Rt of the compounds in the 10 blank meconium samples nor in the samples fortified with common drugs of abuse, including morphine, codeine, 6-acetylmorphine, methadone, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine, amphetamine, methamphetamine, 3,4-methylendioxyamphetamine, 3,4-methylendioxymethamphetamine, 3,4-methylendioxyethylamphetamine, cocaine, benzoylecgonine, ecgoninemethylester, cocaethylene, lysergic acid diethylamide, ketamine, norketamine, gammahydroxybutyric acid, nicotine and cotinine.

The linearity was verified through 5 calibration curves analyzed in 5 different days. The straight-line fit was performed by linear regression, applying 1/x weighting factor over the concentration range of LOQ to 400 ng/g. For all the analytes, mean r^2 was $\geq$0.99. To guarantee the correct Rt for each analyte, a mobile phase was injected every 4 injections to clean the analytical column. The LOQ was 5 ng/g for all the compounds, except for bromazepam and norfluoxetine (10 ng/g) and for lorazepam, desmethylvenlafaxine and norsertraline (20 ng/g). Imprecision and accuracy for lorazepam, desmethylvenlafaxine and norsertraline were only evaluated at two QC concentrations (30 ng/g and 150 ng/g), as the lowest QC concentration (15 ng/g) was below their LOQ. Fig. 1A and 1B show the chromatogram of the main MRM transition for each antidepressant and benzodiazepine, respectively, at the LOQ. The LOD was 20 ng/g for lorazepam, desmethylvenlafaxine and norsertraline; 10 ng/g for norfluoxetine; 5 ng/g for bromazepam, clonazepam, lormetazepam, oxazepam, α-OH-alprazolam, 7-aminoflunitrazepam and venlafaxine; 2 ng/g for triazolam; and of a 1 ng/g for the rest.

Results for imprecision and accuracy are shown in Table S2 (Supplementary Data). Accuracy was satisfactory for all the analytes (90.6-111.5% of target concentration).

Intra-assay, inter-assay and total imprecision were <14.6%, <10.1% and <14.6%, respectively.

Matrix effect, and extraction and process efficiency results are shown in Table 2. Matrix effect was negligible for 11 compounds; ion suppression was observed for 10 compounds (desipramine, norclomipramine, fluoxetine, sertraline, norsertraline, desmethylvenlafaxine, citalopram, desmethylcitalopram, midazolam, 7-aminoflunitrazepam), ranging from -73% to -22.5% (%CV= 5.7-19.9%); and 8 compounds (bromazepam, α-OH-alprazolam, oxazepam, alprazolam, triazolam, lorazepam, nordiazepam, lormetazepam) showed ion enhancement from 28% to 194.9% (%CV= 2.4-21.4%). The behavior of the deuterated analogues was similar (-40.6% to -23% for those IStd showing ion suppression; 46.3 to 198.1% for those IStd showing signal enhancement; %CV= 5.3-22.7%), compensating matrix effect on linearity, imprecision and accuracy. Extraction efficiency ranged from 35.9% to 91.2% (%CV= 3.7-34.7%), and process efficiency from 20.1% to 188.2%.

All analytes were stable for 72 h in autosampler at 6°C, with a %loss ranging from -8.5% to 9%. In addition, all the analytes were stable after 3 freeze/thaw cycles (%loss= -1.2% to -18.9%), except desmethylcitalopram and 7-aminoflunitrazepam, with a loss lower than -47% and -39.6%, respectively.

Hydrolysis assessment

Available positive specimens (n=4) were employed to evaluate the effect of sample hydrolysis. These authentic samples tested positive for clonazepam, alprazolam, α-OH-alprazolam, diazepam, nordiazepam, fluoxetine, norfluoxetine, clomipramine and norclomipramine. Concentrations were similar after performing the enzymatic or alkaline

hydrolysis and when no hydrolysis was performed (%CV= 1.8%-15%, Table 3). Hydrolysis was not performed for method validation.

Application to real specimens

As a proof of the method, four meconium specimens from newborns whose mothers were on antidepressant or benzodiazepine treatment during pregnancy were analyzed. Meconium tested positive for alprazolam (n=1, 4.5 ng/g), α-hydroxyalprazolam (n=1, 6.5 ng/g), diazepam (n=1, 17.1 ng/g), nordiazepam (n=1, 32.1 ng/g), clonazepam (n=1, 5.1 ng/g), fluoxetine (n=2, >400 ng/g), norfluoxetine (n=2, >400 ng/g), clomipramine (n=1, 321.1 ng/g) and norclomipramine (n=1, 85.1 ng/g). Metabolites concentrations were higher to those found for the parent drug (1.1 to 2.7-fold higher), except for norclomipramine in Specimen 2, for which concentration was around 3.8 times lower than that for clomipramine. Fig. S1, S2, S3 and S4 (Supplementary Data) show the chromatograms of the two MRM transitions of the detected analytes and corresponding IStd in Specimen 1, Specimen 2, Specimen 3 and Specimen 4, respectively.

Discussion

This study describes a LC-MS/MS method for the identification and quantification of 15 antidepressants and 14 benzodiazepines in meconium, for the first time including bromazepam, flunitrazepam and triazolam. The same sample aliquot (0.25 g) was extracted for the analysis of all the analytes, but two LC-MS/MS injections were performed for the determination of both, antidepressants and benzodiazepines. Although a different chromatographic gradient was employed in each case, both methods shared the same sample pretreatment, mobile phase composition and analytical column, so that analysis of all the compounds could be programmed consecutively without the need of

doing any handling by the operator. Moreover, meconium specimens can be analysed for just one or both groups of drugs depending on the specific request.

In meconium not only the parent drug but also the metabolites are expected. For this reason, we included in the method the major metabolite for many of the compounds (nortriptyline, desipramine, norclomipramine, norfluoxetine, norsertraline, desmethylvenlafaxine, desmethylcitalopram, α-hydroxyalprazolam, nordiazepam and 7-aminoflunitrazepam) and evaluated the effect of alkaline or enzymatic hydrolysis on drug concentrations. In the positive real samples, the corresponding metabolites were always detected, usually at higher concentrations. The analysis of real positive samples with and without hydrolysis showed no differences in drug concentrations for any of the analytes present in the specimens (clonazepam, alprazolam, α-OH-alprazolam, diazepam, nordiazepam, fluoxetine, norfluoxetine, clomipramine and norclomipramine). Conjugation of the above mentioned pharmaceuticals or their metabolites (mostly with glucuronic acid) is described in the literature (32). However, we did not find differences in the concentrations observed with and without hydrolysis; this could be due to the fact that glucuronidation of the parent drug or the metabolites included in the present method is a minor metabolic pathway, linked to a low transplacental passage of the glucuronides. Moreover, although the enzyme responsible for the glucuronidation of these compounds is already expressed in the fetus, its activity is low (33,34). Assuming that a similar metabolic profile is expected for the other antidepressants and metabolites included in the method (32), we decided to avoid the hydrolysis step. Applying the proposed procedure, the method was fully validated, obtaining satisfactory results for all the studied parameters.

Few authors previously reported methods for the detection of benzodiazepines in meconium (16,19-22) while only one reported the detection of antidepressants (16).

Moreover, most of them included a limited number of compounds or even did not indicate the specific drugs included. Ostrea et al. (19) analyzed 98 meconium samples for illicit drugs and other xenobiotic agents using a screening method by HPLC-UV and another by GC-MS (1 g of meconium, LOD 200 ng/g). Among the compounds of interest in the present work (antidepressants and benzodiazepines), Ostrea et al. only reported the detection of sertraline in 3.1% of the cases. Bar-Oz et al. (20) analyzed 185 meconium specimens for drugs of abuse and some medicines, including benzodiazepines, by immunoassay using 0.2 g of meconium, with a LOD of 50 ng/g; the authors stated that when a positive result was detected, confirmation by GC-MS was performed; however, they reported 2 positive cases for benzodiazepines but the specific drugs detected were not indicated. García-Algar et al. (21) published a case report of a newborn whose mother used alprazolam throughout the pregnancy, which was confirmed by the analysis of different neonatal matrices, including meconium; their analytical method (1 g of meconium, LOD of 7 ng/g and LOQ of 20 ng/g) allowed the determination of alprazolam and α-OH-alprazolam. Marin et al. (22) and Pichini et al. (16) described the more extensive methods for the determination of benzodiazepines and/or antidepressants in meconium. Marin et al. (22) measured 13 benzodiazepines and metabolites (alprazolam, α-OH-alprazolam, clonazepam, 7-aminoclonazepam, diazepam, nordiazepam, lorazepam, midazolam, oxazepam, temazepam, desalkylflurazepam, α-OH-ethylflurazepam and α-OH-triazolam) in 1 g of meconium using LC-MS/MS. The method was validated, but it was not applied to real meconium samples. The achieved LODs and LOQs were 10 ng/g and 20 ng/g, respectively, for all the analytes, similar to our LOD and LOQ values, but employing 1 g of sample instead of 0.25 g. They applied enzymatic hydrolysis (with β-glucuronidase) to meconium samples fortified with oxazepam glucuronide, and hydrolysis efficiency was verified by recovering 100.4% of the corresponding oxazepam concentration. However, as indicated above, applying the

same enzymatic hydrolysis we did not find an increase in parent drugs or metabolites concentrations in real samples containing different antidepressants and benzodiazepines. Finally, Pichini et al. (16) developed a LC-MS/MS method for the determination of 18 antidepressants, anxiolytics and antipsychotic drugs (duloxetine, venlafaxine, citalopram, paroxetine, clomipramine, amitriptyline, imipramine, fluoxetine, sertraline, medazepam, oxazepam, lorazepam, alprazolam, clonazepam, diazepam, lormetazepam, clozapine and quetiapine), including 4 metabolites (norfluoxetine, norsertraline, nordiazepam, norclozapine) in meconium. The sample (0.5 g) was homogenized with methanol and evaporated, and was back-extracted with ethylacetate:ACN (80:20, v/v) in basic conditions. No hydrolysis was applied to the meconium samples and they did not report the investigation of sample hydrolysis effects on drug concentrations. Their LOD ranged from 1.5 to 8 ng/g and the LOQ from 5 to 25 ng/g, similar to the values obtained in our method, but using the double of sample amount. That method was applied to the analysis of 11 real samples from newborns whose mothers reported the use of these pharmaceuticals during pregnancy, and in-utero exposure to the drug was confirmed in 6 cases. The drugs detected were paroxetine, sertraline, norsertraline, fluoxetine, citalopram, lorazepam, diazepam, nordiazepam, oxazepam and clonazepam, at concentrations ranging from the LOD to 6659.8 ng/g in their analysis. None of the published methods, as mentioned above, included bromazepam, flunitrazepam or triazolam. Therefore, the present LC-MS/MS method is the first one that allows the determination of these four drugs in meconium.

As indicated before, in our method we only employed 0.25 g of meconium for the determination of all the compounds, still achieving similar sensitivity to that in previously reported methods, where the amount of meconium used was 0.5 g or 1 g. As usually the amount of specimen is limited, the ability of using less sample amount becomes critical when the determination of the most common drugs of abuse is requested. The use of a

small sample amount is critical, as usually several analyses should be performed for the same real case. So, when the determination of the most common drugs of abuse is requested, it may not be enough specimen available for other analysis.

Finally, one aspect to be aware of when the specimen has been submitted to several freeze/thaw cycles is the loss of almost half of the concentration for the metabolites desmethylcitalopram and 7-aminoflunitrazepam. However, their respective parent drugs (citalopram and flunitrazepam) proved to be stable in those conditions and, therefore, positivity of the specimen could be confirmed by the identification of the parent drug. Wu et al. (35) also evaluated the stability of benzodiazepines in fortified meconium samples, and showed that those included in our method were stable after 15 days of storage at 4°C and at room temperature, but some (lorazepam, oxazepam and nordiazepam) were unstable at 37°C. Nevertheless, they did not include any freeze/thaw cycle in their study.

Conclusion

The method described in this manuscript allows the determination of the most common antidepressants and benzodiazepines, and most of their main metabolites, in meconium in two sequential LC runs, with enough sensitivity to be used in the clinical and forensic practice. The main improvements of our method were the inclusion of the main metabolite for many of the compounds, the inclusion for the first time of bromazepam, flunitrazepam and triazolam, and the smallest amount of sample employed for the determination of all the compounds compared to previously published methods, and still achieving a similar sensitivity.

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Table 1. MRM transitions, cone voltage (CV), collision energy (CE) and retention time (Rt) for the Antidepressants (ADS), the Benzodiazepines (BZD) and the deuterated analogues. The MRM transition selected for quantification is underlined.

ADS	MRM transition	CV (V)	CE (eV)	Rt (min)	IStd	BZD	MRM transition	CV (V)	CE (eV)	Rt (min)	IStd
Desmethylvenlafaxine	<u>264.5>57.4</u> 264.5>246.4	30 30	15 13	1.30	Venlafaxine-d_3	Zolpidem	<u>308.3>235.3</u> 308.3>263.2	50 50	34 26	1.30	Zolpidem-d_6
Venlafaxine	<u>278.5>57.4</u> 278.5>260.5	25 25	17 13	1.45	Venlafaxine-d_3	Midazolam	<u>326.4>291.3</u> 326.4>223.3	45 45	25 37	1.50	Zolpidem-d_6
Desmethylcitalopram	<u>311.3>108.8</u> 311.3>262.3	30 30	25 20	1.80	Citalopram-d_6	7-Aminoflunitrazepam	<u>284.4>135</u> 284.4>227.2	45 45	29 25	1.80	7-Aminoflunitrazepam-d_7
Citalopram	<u>325.5>108.8</u> 325.5>262.4	40 40	23 19	1.80	Citalopram-d_6	Bromazepam	<u>318.1>182.2</u> 318.1>209.2	45 45	34 26	2.55	Zolpidem-d_6
Desipramine	<u>267.4>71.5</u>	25	15	2.25	Desipramine-d_3	α-OH-Alprazolam	<u>325.1>279.1</u>	45	20	3.60	α-OH-

	267.4>208.3	25	23				325.1>297.1	45	30		Alprazolam-d_5
Imipramine	281.3>85.9 281.3>57.8	30 30	16 40	2.50	Imipramine-d_3	Oxazepam	287.3>241.3 287.3>269.3	35 35	21 15	4.65	Oxazepam-d_5
Paroxetine	330.2>69.5 330.2>192.2	45 45	26 22	2.55	Paroxetine-d_6	Alprazolam	309.3>281.3 309.3>205.2	50 50	25 39	4.75	Alprazolam-d_5
Amitriptyline	278.3>90.9 278.3>105	35 35	20 22	2.60	Imipramine-d_3	Triazolam	343.4>308.4 343.4>315.3	55 55	25 29	5.25	Alprazolam-d_5
Nortriptyline	264.3>233.2 264.3>90.9	30 30	14 2	2.70	Nortriptyline-d_3	Lorazepam	321.4>275.3 321.4>303.3	35 35	25 13	5.35	Lorazepam-d_4
Norfluoxetine	296.2>133.8 296.2>29.8	20 20	6 10	3.00	Norfluoxetine-d_6	Nordiazepam	271.1>139.8 271.1>164.9	45 45	26 28	5.95	Nordiazepam-d_5
Fluoxetine	310.2>43.8 310.2>148.1	25 25	12 8	3.40	Fluoxetine-d_6	Clonazepam	316.1>270 316.1>213.9	45 45	24 42	6.20	Clonazepam-d_4
Norsertraline	292.2>275.3	15	10	3.55	Sertraline-d_3	Flunitrazepam	314.1>268	45	26	6.65	Flunitrazepam-d_7

	292.2>159	15	20				314.1>239.1	45	34		
Norclomipramine	301.4>71.5 301.4>242.3	30 30	17 23	3.65	Clomipramine-d_3	Lormetazepam	335.4>289.3 335.4>317.3	35 35	25 13	7.70	Diazepam-d_5
Sertraline	306.1>159.1 306.1>275.1	25 25	28 12	3.70	Sertraline-d_3	Diazepam	284.9>154.2 284.9>193.2	45 45	27 33	8.70	Diazepam-d_5
Clomipramine	315.2>85.9 315.2>57.8	30 30	18 44	3.80	Clomipramine-d_3						
Venlafaxine-d_3	284.2>64	25	20	1.50		Zolpidem-d_6	314.3>235.1	50	36	1.30	
Citalopram-d_6	331.4>108.8	35	25	1.80		7-Aminoflunitrazepam-d_7	291.2>137.9	45	28	1.80	
Desipramine-d_3	270.2>75.1	25	15	2.25		α-OH-Alprazolam-d_5	330.1>302.1	45	27	3.55	
Nortriptyline-d_3	267.2>90.5	35	18	2.60		Oxazepam-d_5	292.1>246	35	22	4.55	
Imipramine-d_3	284.3>88.6	30	16	2.50		Alprazolam-d_5	314.1>286	45	26	4.65	
Paroxetine-d_6	336.2>75.5	45	28	2.55		Lorazepam-d_4	325.1>278.9	30	26	5.25	

Norfluoxetine-d_6	302.2>140.1	15	7	3.00		Nordiazepam-d_5	276.1>140	50	58	5.75	
Fluoxetine-d_6	316.2>43.5	25	10	3.30		Clonazepam-d_4	320.1>274	45	26	6.05	
Sertraline-d_3	309.3>159	20	25	3.70		Flunitrazepam-d_7	321.1>275	50	26	6.50	
Clomipramine-d_3	318.2>88.7	35	16	3.80		Diazepam-d_5	290.1>153.9	45	24	8.55	

Table 2. Matrix effect (ME), extraction efficiency (EE) and process efficiency (PE) at 30 ng/g and 150 ng/g QC concentrations.

Compound	%ME (%CV) (n=10)		%EE (%CV) (n=6)		%PE (n=6)	
	30 ng/g	150 ng/g	30 ng/g	150 ng/g	30 ng/g	150 ng/g
Amitriptyline	16.4 (6.7)	18.1 (10.5)	66.7 (16.2)	63.3 (15.5)	77.7	74.8
Nortriptyline	-19.9 (8.1)	-18.9 (13)	60.3 (20.3)	55.6 (17.2)	48.3	45.1
Nortriptyline-d_3	-11.3 (17.2)	-16.1 (12.5)	62.8 (17.2)	56.8 (12.5)	55.7	47.7
Imipramine	6.4 (8)	10.2 (9.3)	66.9 (18.8)	66 (12.7)	71.2	72.7
Imipramine-d_3	9.6 (9.1)	8.5 (10.9)	68.5 (16.4)	65.7 (11)	75.1	71.2
Desipramine	-24.5 (12.4)	-17.7 (10.8)	53.3 (26.3)	49 (20.5)	40.3	40.3
Desipramine-d_3	-23 (13.2)	-18.1 (11.6)	53.7 (24.7)	43.7 (19.9)	41.4	35.8
Clomipramine	-18.3 (6.9)	-16.5 (13.6)	66.8 (18.3)	60.1 (12.8)	54.6	50.2
Clomipramine-d_3	-11.3 (6.9)	-12.5 (14.7)	67.2 (16.3)	60.7 (12.4)	59.6	53.1
Norclomipramine	-40.2 (13.3)	-28.7 (19.2)	55.9 (28.9)	48.3 (21.2)	33.4	34.5
Fluoxetine	-18.2 (16.8)	-25 (19.9)	69.7 (29.8)	55.1 (34.7)	57	41.3
Fluoxetine-d_6	-10.4 (13.4)	-19.8 (22.7)	70.2 (19)	56.5 (23)	62.9	45.4
Norfluoxetine	-2.2 (7.2)	-4.1 (8.1)	60.5 (29.1)	57.4 (16.7)	59.1	55
Norfluoxetine-d_6	-0.3 (8.1)	-3.3 (9.4)	61.7 (27.8)	58.2 (15.7)	61.5	56.3
Sertraline	-29.3 (10.7)	-30.6 (14)	62.9 (28)	53.4 (22.7)	44.5	37.1

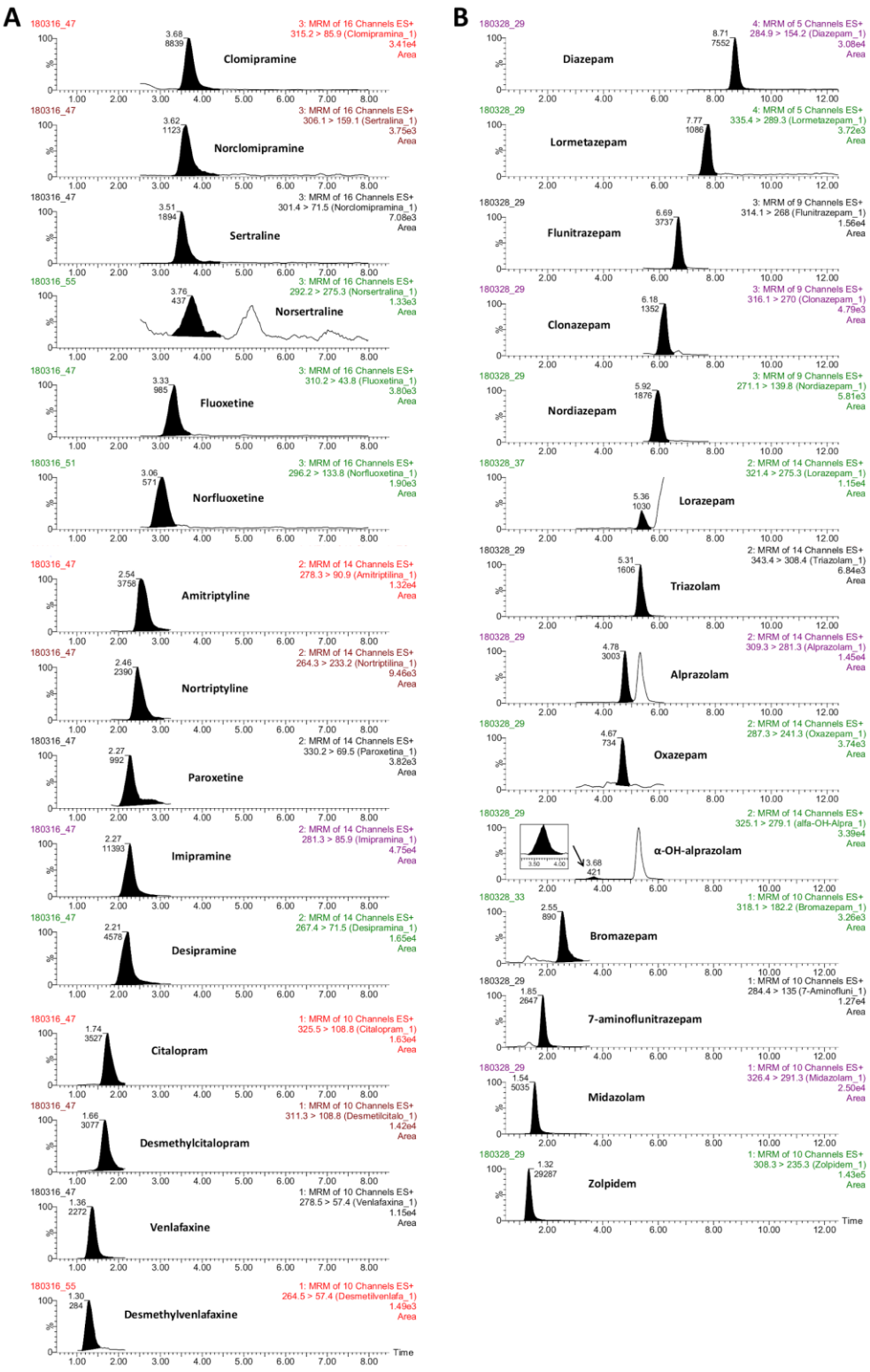
Sertraline-d_3	-27.9 (11.8)	-32 (19.8)	63 (24.8)	52.1 (21)	45.5	35.4
Norsertraline	-31.6 (17.9)	-15.4 (12.3)	57.2 (33.8)	59.1 (24.5)	39.1	49.9
Paroxetine	-10.5 (6.5)	-8.3 (6)	55.1 (18.4)	54.7 (13.8)	49.3	50.2
Paroxetine-d_6	-7 (7.8)	-8.7 (6.5)	54.7 (20.3)	51.1 (11.8)	50.9	46.7
Venlafaxine	-5.6 (6.9)	14 (6.6)	72 (9.7)	78 (7.9)	68	88.9
Venlafaxine-d_3	-4.5 (7.4)	10.6 (6.9)	74.1 (9)	77.2 (5.4)	70.8	85.4
Desmethylvenlafaxine	-73 (14.6)	-66.4 (16.5)	74.2 (17.8)	71.6 (12.8)	20.1	24
Citalopram	-24.5 (5.7)	-19.2 (8.7)	70.6 (14.4)	67.1 (12.2)	53.3	54.2
Citalopram-d_6	-23.1 (6.7)	-19.7 (9)	72.1 (14.1)	66.5 (8.8)	55.4	53.4
Desmethylcitalopram	-38.7 (10.5)	-27.8 (10.5)	43.2 (19.8)	35.9 (14)	26.5	25.9
Alprazolam	54.1 (2.4)	28.1 (4.4)	63.7 (9.1)	60.6 (13.2)	98.2	77.7
Alprazolam-d_5	46.3 (4.1)	19.2 (6.4)	72.1 (13.9)	61.8 (12.1)	105.5	73.6
α-OH-alprazolam	137.4 (8.8)	78.9 (16.5)	76.2 (19.5)	77.8 (18)	180.9	139.1
α-OH-alprazolam-d_5	165.2 (7.7)	87.4 (15.6)	75.4 (18.8)	77.1 (16.8)	199.9	144.5
Bromazepam	142.3 (7.5)	87.6 (21.4)	71.5 (7.9)	74.7 (24.1)	173.1	140.1
Clonazepam	9.5 (3.9)	3.9 (4.9)	76.1 (10.8)	76.7 (7.2)	83.3	76.7
Clonazepam-d_4	7.9 (3.2)	1.2 (4.5)	79.6 (12.6)	78.7 (5.3)	85.9	79.6
Diazepam	2.2 (11.1)	8.1 (8)	81.5 (13.3)	73.3 (14.7)	83.3	79.3
Diazepam-d_5	12.6 (6.6)	10.9 (6.6)	83.2 (7.1)	74.8 (7.9)	93.7	82.9

Nordiazepam	95.9 (9.3)	62 (13.9)	83 (17.9)	91.2 (12.9)	162.6	147.8
Nordiazepam-d_5	110.2 (11.2)	75.2 (18.5)	81.3 (17.7)	92.6 (15.1)	170.8	162.3
Flunitrazepam	2.1 (7.6)	-1 (8.7)	77.5 (10.4)	65.8 (9.9)	79.1	65.1
Flunitrazepam-d_7	-0.3 (6.5)	1.2 (8.5)	88.4 (15.9)	65.8 (8.6)	88.2	66.6
7-aminoflunitrazepam	-42.9 (9.4)	-38.7 (13.9)	49.1 (9.5)	44.6 (27.9)	28	27.3
7-aminoflunitrazepam-d_7	-40.6 (9.8)	-40.6 (12.9)	51.9 (11.4)	45 (27.2)	30.8	26.7
Lorazepam	184.1 (17.3)	97.6 (19.8)	57.2 (23.5)	64.6 (22.3)	162.4	127.7
Lorazepam-d_4	148 (11.2)	85.3 (20.9)	74.6 (15.4)	64.5 (21.9)	184.9	119.5
Lormetazepam	194.9 (9.6)	115.9 (12.2)	59.5 (14)	66.3 (17.7)	175.5	143.2
Midazolam	-22.5 (6.6)	-21.2 (7.2)	63.2 (9.6)	60.4 (12.6)	49	47.6
Oxazepam	154.1 (10.3)	81.3 (14.8)	74.1 (20.7)	82.6 (14.4)	188.2	149.8
Oxazepam-d_5	198.1 (10)	106.9 (16.5)	80.6 (24.6)	80.4 (14.9)	240.4	166.4
Triazolam	30.9 (16.5)	5.3 (13.2)	46 (13)	52.5 (23.8)	60.3	55.3
Zolpidem	-10.1 (4)	- 11.7 (5.6)	77.2 (3.7)	76 (9.8)	69.4	67.2
Zolpidem-d_6	-4.3 (4.7)	-11.8 (4.6)	78.1 (4.7)	77.1 (4.6)	74.8	68

Table 3. Concentrations found in 4 positive specimens with and without hydrolysis

Specimen	Compound	Without Hydrolysis (ng/g)	Basic Hydrolysis (ng/g)	Enzymatic Hydrolysis (ng/g)	%CV
1	Clonazepam	5.1	6.9	6	15
	Norfluoxetine	2720.5	3566.4	3400.1	13.9
	Fluoxetine	996	1113.7	972.7	7.4
2	α-OH-alprazolam	6.5	6.7	6.5	1.8
	Alprazolam	4.5	4.6	4.3	3.4
3	Norclomipramine	85.1	76.4	76.4	6.3
	Clomipramine	321.1	296.7	305.5	4
	Diazepam	17.1	20.3	20	9.2
	Nordiazepam	32.1	36.3	32.4	7
4	Norfluoxetine	1817	2170.2	1955.8	9
	Fluoxetine	1681.8	1717.1	1476.2	8

Fig. 1 MRM chromatograms of the quantifier transition in a blank meconium sample fortified at the LOQ for the Antidepressants (A) and for the Benzodiazepines (B).



Supplementary Data

Table S1. Parameters evaluated for method validation and acceptance criteria

PARAMETER	METHOD	ACCEPTANCE CRITERIA
Selectivity	Endogenous interferences: 10 different blank meconium samples after addition of the IStd Exogenous interferences: Blank meconium samples fortified with other common drugs of abuse and medicines at 1000 ng/g	NO interferences
Linearity	Calibration curves (n=5) with 5 to 7 calibration levels.	$r^2 \geq 0.99$ Calibrators' residuals ±20%.
Accuracy	At low, medium and high QC concentrations, 5 different days (n=15)	80-120% of the nominal concentration
Intra-assay, inter-assay and total imprecision	At low, medium and high QC concentrations, 5 different days (n=15)	CV<20%[a]

LOD	Blank meconium samples fortified at decreasing concentrations, [2 replicates, 3 different days (n=6)]	Two MRM transitions detected with a s/n>3, and with adequate ion ratio[b]
LOQ	Blank meconium samples fortified at the lowest calibration level [3 replicates, 3 different days (n= 9)]	Quantification with acceptable accuracy (80-120% of the nominal concentration) and precision (%CV<20%)
Matrix effect	At 30 ng/g and 150 ng/g by comparing average analyte peak area in blank meconium samples (n=10, from different neonates) fortified after extraction with average peak area when the analyte was directly prepared in the eluent solvent (neats, n=6)[c]	-

Extraction efficiency	At 30 ng/g and 150 ng/g by comparing average peak area in blank meconium samples fortified before extraction (n=6) with average peak area in blank meconium samples fortified after extraction (n=10)[c]	-
Process efficiency	At 30 ng/g and 150 ng/g by comparing average peak area in blank meconium samples fortified before extraction (n=6) with average peak area of neats (n=6)[c]	-
Autosampler stability	At low, medium and high QC levels by comparing concentrations of fresh QC samples (n=3) with those obtained after 72 h in the autosampler at 6 °C.	%loss <20%
Freeze/thaw stability	At low, medium and high QC concentrations by comparing mean concentration after 3 freeze/thaw cycles (n=3) with mean concentration of freshly prepared QCs (n=3)	%loss <20%

[a] Calculated according to: Krouwer and J.S., Rabinowitz R. (1984) How to improve estimates of imprecision. *Clinical Chemistry,* **30**, 290-292.

[b] Ion ratio acceptance criteria according to European Union Decision, 2002/657/EC (2002). Commission Decision of 12 August 2002 Implementing

Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *The Official Journal of the European Communities*, **221**, 8-36

[c] Calculated according to: Matuszewski B.K., Constanzer M.L., Chavez-Eng C.M. (2003) Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, **75**, 3019-3030

Table S2. Imprecision and accuracy in meconium at 15 ng/g, 30 ng/g and 150 ng/g QC concentrations.

Compound	Intra-assay imprecision (n=15; %CV)			Inter-assay imprecision (n=15; %CV)			Total imprecision (n=15; %CV)			Accuracy (n=15; % target concentration)		
	15 ng/g	30 ng/g	150 ng/g	15 ng/g	30 ng/g	150 ng/g	15 ng/g	30 ng/g	150 ng/g	15 ng/g	30 ng/g	150 ng/g
Amitriptyline	5	3.1	3	8.4	6.8	5.7	9.8	7.5	6.5	99.7	97.5	93.1
Nortriptyline	4.5	3.5	2.9	6.7	6.6	7.3	8	7.5	7.9	100	96.8	94.3
Imipramine	2.8	2.1	2.8	2.9	0	1.8	4	2.1	3.3	106.2	104.1	100.3
Desipramine	3.8	3	2.6	0	0	3.7	3.8	3.8	4.5	111.1	110.4	109
Clomipramine	2.1	2.6	2.2	6.4	5.9	7	6.8	6.4	7.3	110.3	107.9	107.5
Norclomipramine	4.9	4.9	4.3	4.4	6.7	10.1	6.6	8.3	11	100.3	103.5	102.5
Fluoxetine	3.1	3.3	1.9	3.3	3.9	3.4	4.5	5.1	3.9	108.2	105.9	104.2
Norfluoxetine	14.6	3.1	6.4	0	7.3	2.7	14.6	7.9	6.9	111.5	106.5	106.5
Sertraline	6	4.1	1.9	0	8.1	4.5	6	9.1	4.9	106.8	106.3	106.1
Norsertraline	-	5.9	7.9	-	6.2	6.8	-	8.6	10.5	-	108.5	104.2
Paroxetine	4	3	2.5	0	4	3.3	4	5	4.1	108.5	107.6	103.7
Venlafaxine	3.2	2.9	2.2	2.1	3.2	3.4	3.9	4.3	4	104.3	103.4	100.7
Desmethylvenlafaxine	-	2.2	5	-	3.2	6.6	-	3.9	8.3	-	111.1	101.1
Citalopram	2.9	2.4	2.5	3.1	5.5	4.1	4.3	6	4.9	109.2	107.9	105.1
Desmethylcitalopram	6.9	5.7	5.6	2.7	7.5	6.1	7.4	9.5	8.3	101.3	101.4	102.5
Alprazolam	2	2.4	4.7	5.8	0	1.9	6.2	2.4	5.1	104	98.6	96.8
α-OH-alprazolam	3.9	3.6	2.1	4.9	1.5	3.9	6.3	3.9	4.4	108.8	103	102.1

Bromazepam	6.1	5.2	5.9	4.8	8.4	2.9	7.8	9.9	6.6	106.6	95.9	90.6
Clonazepam	2.4	2.6	2.4	3.8	2	0	4.4	3.2	2.4	106.9	102.5	101.9
Diazepam	3	2	1.6	3.2	4.2	3.9	4.4	4.7	4.2	109.7	108.4	105.9
Nordiazepam	3.2	1.4	1.2	4.6	4.9	5.3	5.6	5.1	5.5	107.8	104.8	100.9
Flunitrazepam	3.5	2.3	4.5	0	0	0	3.5	2.3	4.5	106.8	102.5	101.5
7-aminoflunitrazepam	2.8	2.2	1.5	5	3	4.8	5.7	3.7	5.1	106.4	100.2	100
Lorazepam	-	8.5	5.4	-	0	0	-	8.5	5.4	-	101.2	108.6
Lormetazepam	4.3	4.7	5.7	9.3	8.3	6.9	10.3	9.5	9	101.2	98.8	97.9
Midazolam	7.8	7.5	5.5	7.6	3.6	2.4	10.9	8.3	6	105.8	101.2	107.7
Oxazepam	3.3	3.1	5	0	0	0	3.3	3.1	5	110.8	106.5	105.5
Triazolam	8	4.1	7.8	2.9	2	0	8.5	4.5	7.8	105.5	104.1	103.5
Zolpidem	1.8	2.6	1.6	5.4	1.3	2.8	5.7	2.9	3.2	105	98.6	98.1

Fig. S1. Chromatograms of the two MRM transitions for clonazepam, fluoxetine, norfluoxetine and their respective internal standards in a blank meconium sample (A) and in meconium specimen 1 (B).

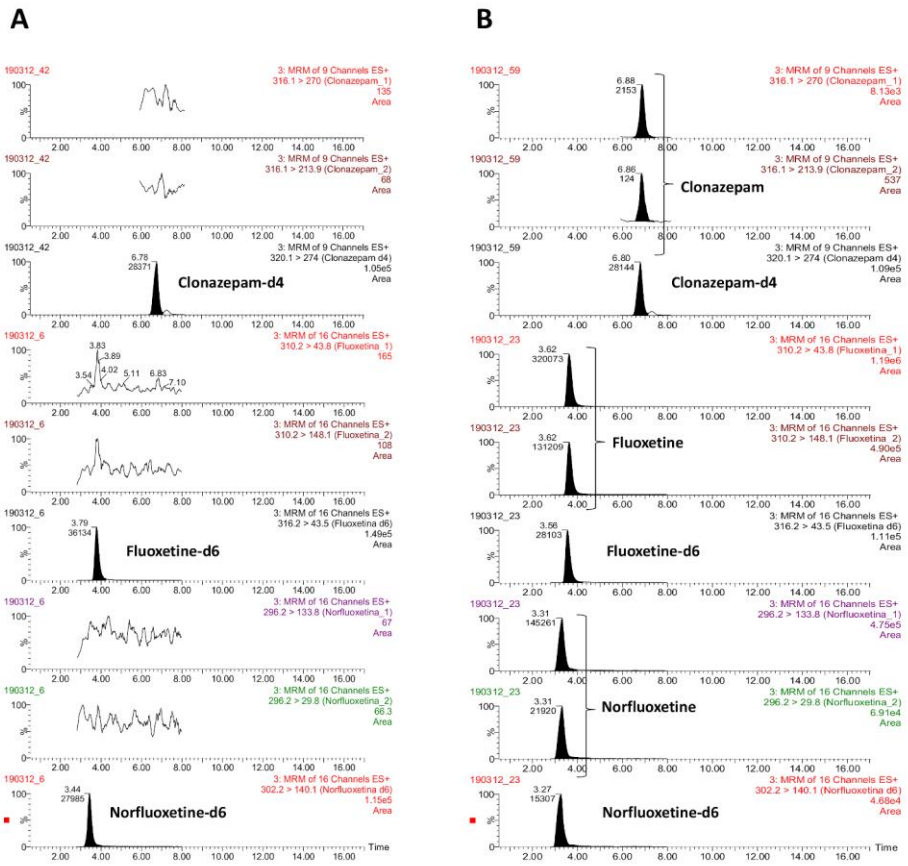


Fig. S2. Chromatograms of the two MRM transitions for α-hydroxyalprazolam, alprazolam and their respective internal standards in a blank meconium sample (A) and in meconium specimen 2 (B).

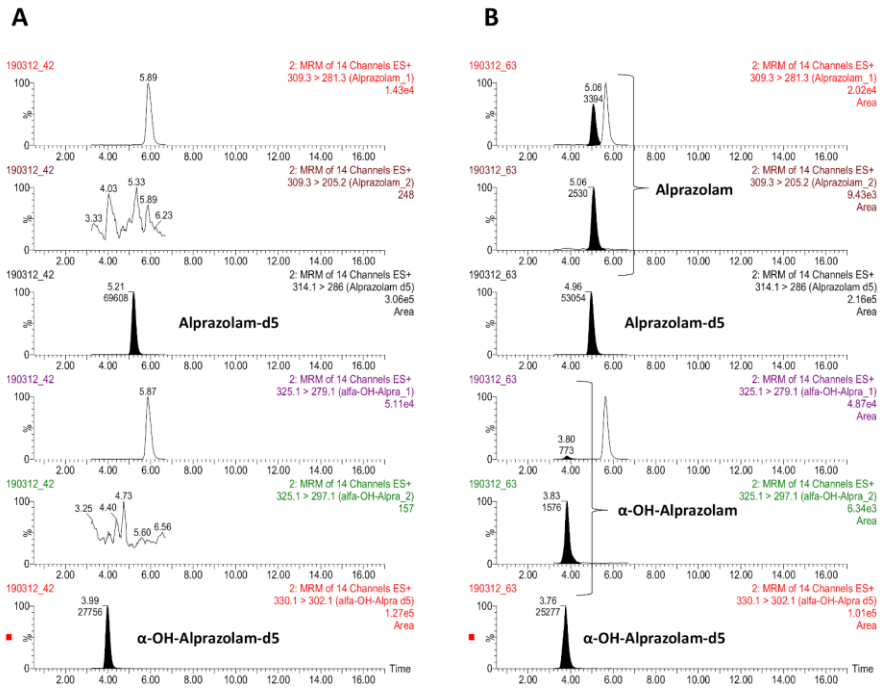


Fig. S3. Chromatograms of the two MRM transitions for norclomipramine, clomipramine, diazepam, nordiazepam and their respective internal standards in a blank meconium sample (A) and in meconium specimen 3 (B).

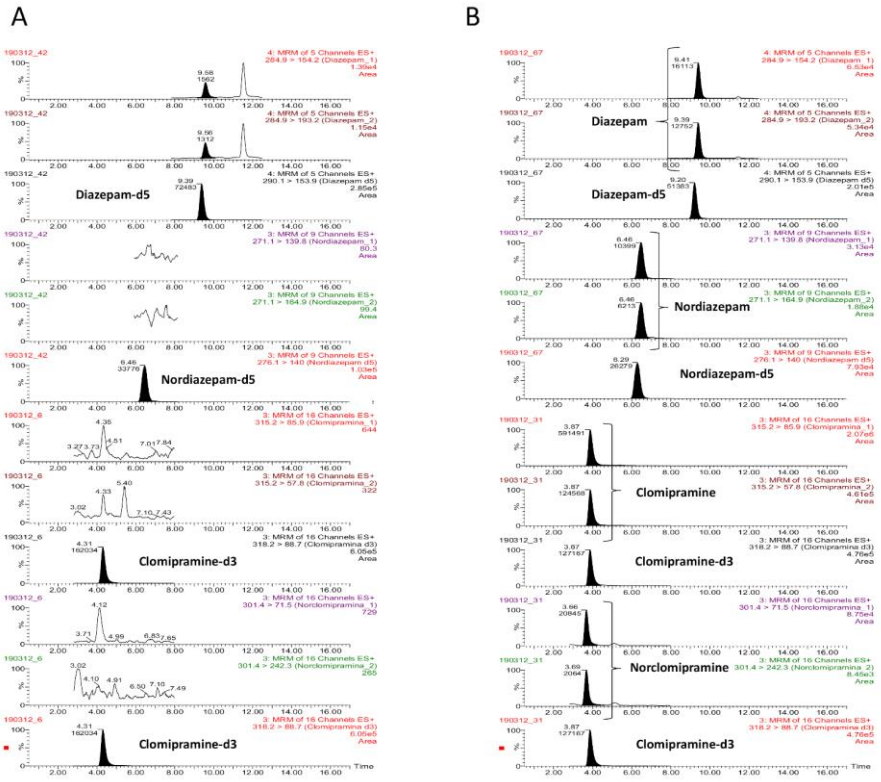


Fig. S4. Chromatograms of the two MRM transitions for fluoxetine, norfluoxetine and their respective internal standards in a blank meconium sample (A) and in meconium specimen 4 (B).