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Simultaneous analysis of frequently used licit and illicit psychoactive drugs in breast milk by liquid chromatography tandem mass spectrometry

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ABSTRACT

A liquid chromatography tandem mass spectrometry (LC–MS–MS) method for the quantification of frequently used licit (caffeine, nicotine and cotinine) and illicit drugs (opiates, cocaine, cannabinoids and amphetamines) in breast milk was developed and fully validated. Chromatography was performed on a reverse-phase column using a gradient of 2 mM ammonium acetate, pH 6.6, and methyl alcohol as mobile phase at a flow rate of 0.35 mL/min. Separated analytes were quantified by electrospray ionization tandem mass spectrometry in positive ion mode using multiple reaction monitoring.

Milk samples were kept at −20 °C until analysis and the compounds under investigation were extracted from the matrix by Bond Elut Certify cartridges. The concentration range covered was LOQ to 1000 ng/mL for all the investigated drugs. Intra- and inter-assay imprecision was less than 20%, analytical recovery ranged between 51.6% and 86.5%, matrix effect between 71.1% and 116.6% and process efficiency between 46.8% and 84.0%. Analytes were stable after three freeze–thaw cycles, after 6 months at −20 °C and after the pasteurization process (differences to the initial concentration always lower than 10%). matrix effect ranged from 77.6% to 116.6%, recovery from 51.6% to 86.5%, and process efficiency from 46.8% to 79.0%.

This LC–MS–MS assay was applied to screen samples from the largest Spanish milk bank and samples coming from drug addicted mothers. The developed method provided adequate sensitivity and performance characteristics to prove the presence of only caffeine in a small percentage of samples from milk donating nursing mothers and the presence or absence of most commonly used illicit drugs in breast milk from addicted lactating mothers.

1. Introduction

Breastfeeding is an essential physiological process with health and social benefits [1,2]. Human milk provides nutrition, digestive enzymes, immunological factors of many types, growth factors, hormones, and other bioactive factors [2] and it is widely accepted that breastfed infants have a lower risk of developing necrotizing enterocolitis, enteritis, otitis media, sudden infant death syndrome, lower respiratory tract infections, respiratory syncytial virus infection, insulin-dependent diabetes mellitus, and allergies [3–5]. To protect nursing infants from undesired effects of maternal consumption of any licit or illicit drug, but also to allow effective pharmacologic treatment of breastfeeding mothers, information on drugs (and or metabolites) excretion in human milk is essential [1,6,7]. This is of major importance when breastfeeding newborns with milk coming from milk banks. Indeed, although mother's own milk is clearly the best choice, human milk banking has a long tradition in many countries and has a well recognized role in the care of preterm and sick infants [2].

When drugs are administered to a lactating mother, a certain percentage of the drugs may be excreted into the breast milk [7]. The amount of drug excreted from plasma into breast milk depends on the characteristics of the drug, such as plasma protein binding, ionization, degree of lipophilicity and molecular weight. In general, low plasma protein binding, low molecular weight, high lipophilicity, low pH and high lipid content contribute to the excretion phenomenon [1]. The excretion of drugs in breast milk occurs mostly via passive diffusion, but carrier-mediated transport also occurs for certain drugs [7]. As a result of the infant's small size and the difference in metabolism between infants and their mothers, occasionally this transfer of medication can prove to be harmful to the infant [8–10]. For this reason, drug therapies tend to be limited or strictly controlled during breastfeeding. Furthermore, nursing mothers are recommended to stop or at least

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to reduce tobacco smoking and coffee drinking and to absolutely avoid consumption of drugs of abuse [1]. Despite these warnings, some lactating mothers keep on maintaining their toxic habits so that the presence of significant amounts of most frequently consumed licit and illicit psychoactive drugs in breast milk cannot be excluded. Consequently, both in the case of nursing mothers suspected of drug abuse and more importantly in the case of human milk banks, screening for such substances before breastfeeding protect the newborn from undesired ingestion of potentially harmful compounds.

Breast milk is an unconventional matrix that has been used to assess neonatal acute exposure to drugs, and its main advantage is its easy and non-invasive collection. However, the extraction of drugs from breast milk is an analytical challenge because of its high protein and fat content and changing composition during the postpartum period [1].

Several methods were published for the determination of nicotine [11–16], caffeine [16–19], cannabis [20], cocaine [21,22], amphetamines [23–25], and methadone [26–29] in human milk. Often, only a limited number of substances from the same drug class are included in the assay and in early studies there was no mass spectrometry used to detect the analytes. There is no published evidence of analysis of heroin in human breast milk, while two articles reported the determination of codeine and morphine levels by radioimmunoassay [30] and the determination of morphine and its metabolite by liquid chromatography–ultraviolet spectrophotometry assay [31].

We developed a liquid chromatography tandem mass spectrometry (LC–MS–MS) method to measure licit (tobacco and caffeine) and illicit (opiates, methadone, cocaine, amphetamines and cannabinoids) drugs in human breast milk and applied the validated methodology to screen samples from the largest Spanish milk bank. To our knowledge this is the first LC–MS/MS method to simultaneously quantify 18 drugs and metabolites in breast milk.

2. Materials and methods

2.1. Chemicals and materials

Morphine (MOR), codeine (COD), 6-acetylmorphine (6-MAM), 2-ethylene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), methadone (MTD), cocaine (COC), benzoylecgonine (BZE), cocaethylene (CE), nalorphine (NLP), 11-nor-carboxy-Δ9-tetrahydrocannabinol (THC-COOH), 11-hydroxy-Δ9-tetrahydrocannabinol (THC-OH), Δ9-tetrahydrocannabinol (THC), naphthalen-1-yl-(1-pentylindol-3-yl) methanone (JWH-018), amphetamine (AP), methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA), 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxypropylamphetamine (MDPA) were supplied by LGC Standards Promochem (Milan, Italy). Nicotine (NIC), cotinine (COT) and caffeine (CAF) and N-ethylnorcotinine (NENC) were supplied by Sigma–Aldrich (Milan, Italy). NLP, JWH-C18, MDPA and NENC were used as structurally related internal standards for different drug classes. Deuterated internal standards (MOR-d3, COC-d3, BZE-d3, THC-COOH-d3, AP-d5, MDMA-d5 and COT-d3) were supplied by LGC Standards Promochem (Milan, Italy).

Bond Elut Certify solid-phase extraction (SPE) columns were from Varian (Palo Alto, CA). Ultrapure water and all other reagents of HPLC grade were obtained from Carlo Erba (Milan, Italy).

2.2. Preparation of standard solutions

Stock standard solutions (1 mg/mL) and working solutions (10, 1 and 0.1 μg/mL) of all the analytes were prepared in methyl alcohol and stored at 20 °C until analysis. The internal standards (both structurally related and deuterated ones) working solutions were prepared at a concentration of 10 μg/mL. Calibration standards for all the analytes between LOQ and 1000 ng/mL milk were prepared daily for each analytical batch by adding suitable amounts of methanolic working solutions to 0.5 mL pre-checked drug-free human milk. Quality controls (QC) samples at 850 ng/mL (high control for all analytes under investigation), 400 ng/mL (medium control for all analytes under investigation), and 6 ng/mL (low control for MOR, 6-MAM, COD, MA, BZE, COC, THC, THC-OH, THC-COOH) or 12 ng/mL (low control for COT, CAF, NIC, MDA, AM, MDMA, CE, MTD, EDDP) were prepared in drug-free milk and stored at −20 °C. The QC samples were included in each analytical batch to check linearity, accuracy and precision, and the stability of samples under different storage conditions.

2.3. Sample extraction

To 500 μL breast milk sample (blank, calibrators, QC and real samples) in a glass tube, 500 μL of methyl alcohol were added. The tubes were vortex mixed for 0.5 min and centrifuged at 4000 rpm for 5 min at room temperature. The supernatant was transferred into 15-mL screw-capped glass tubes, diluted with 4 mL of 100 mM ammonium acetate pH 5.5 and applied on a Bond Elut Certify solid-phase extraction (SPE) column, which had been preconditioned with 2 mL methyl alcohol, 2 mL water and 1 mL 100 mM ammonium acetate pH 5.5. The column was further washed with 1 mL 0.1N HCl and dried under vacuum for 5 min. Cannabinoids were eluted with 2 mL methanol, a second elution step with 2 mL dichloromethane:isopropyl alcohol (80:20) with 2% ammonium hydroxide was used for the other analytes. The organic layer was evaporated under nitrogen stream at 30 °C and redissolved in 100 μL of water:methyl alcohol (20:80, v/v).

2.4. Liquid chromatography tandem mass spectrometry

Liquid chromatography tandem mass spectrometry (LC–MS–MS) analyses were performed using an Alliance HPLC system (Waters, Etten-Leur, The Netherlands) interfaced to a Micromass Quattro micro API triple quadrupole mass spectrometer (Waters) equipped with an electrospray (ESI) ion source. Chromatographic separation was achieved at 38 °C with a Zorbax extend C18 column (50 mm × 2.1 mm i.d., 3.5 μm particle size) (Agilent). The gradient was a mixture of solvent A: (2 mM ammonium acetate at pH 6.6) and solvent B: (methyl alcohol) with the following linear program: 0.0 min, 10% B; 0.1–16.0 min: from 10% to 93% B; 16.1–20 min return to initial conditions. The flow rate was kept constant at 0.35 mL/min during the analysis and the sample volume injected was 25 μL.

The tandem mass spectrometer was operated in positive ionization mode with the following parameters: capillary voltage, 3 kV; lens voltage 0.3 V; source temperature, 130 °C; desolvation temperature, 500 °C; cone gas flow rate, 30 L/h; desolvation gas flow rate, 800 L/h. Dry nitrogen (≥99.5%) was used as desolvation and nebulization gas and argon (>99.999%, Praxair, Spain) was used as collision gas. Acquisition was performed in multiple reaction monitoring (MRM) mode and the protonated molecular ion of each compound was chosen as precursor ion. MS and MS/MS spectra of the compounds under investigation were acquired as follows. The compounds dissolved in methyl alcohol at a concentration of 10 μg/mL, were infused through an integrated syringe pump into the ESI probe at a rate of 10 μL/min to tune the mass spectrometer and optimize the acquisition parameters. Cone energy voltages, MRM transitions, and collision energy voltages were established for each analyte and the values are listed in Table 1.

Table 1
LC–MS–MS parameters for the MRM acquisition mode (quantification and confirmation).

Analytes	Retention time (min)	MRM transitions					
		Quantification			Confirmation		
		m/z	CV (V)[a]	CE (eV)[b]	*m/z*	CV (V)[a]	CE (eV)[b]
COT	5.6	177 → 80	25	18	177 → 146	25	18
CAF	6.1	195 → 138	30	20	165 → 110	30	20
NIC	8.5	163 → 132	25	20	163 → 80	25	25
MOR	6.6	286 → 152*	45	5	286 → 165	45	40
6-MAM	9.1	328 → 152*	35	5	328 → 165	35	35
COD	9.5	300 → 152*	47	5	300 → 165	47	43
MDA	6.9	180 → 105	20	11	180 → 163	20	15
AP	7.1	136 → 91	20	15	136 → 119	20	9
MDMA	7.7	194 → 163	20	15	194 → 105	20	23
MA	8.1	150 → 91	20	18	150 → 119	20	10
BZE	7.2	290 → 168	30	25	290 → 105	30	29
COC	11.7	304 → 182	30	25	304 → 82	30	34
CE	12.4	318 → 196	25	20	318 → 168	25	20
EDDP	11.3	278 → 234	50	25	278 → 249	50	35
MTD	13.4	310 → 265	20	15	310 → 105	20	25
THC-COOH	14.8	345 → 327	30	16	345 → 193	30	28
THC-OH	15.7	331 → 193	30	22	331 → 201	30	22
THC	17.5	315 → 193	30	22	315 → 123	30	30
NENC	6.9	191 → 120	25	20			
MDPA	9.3	222 → 163	20	20			
NLR	11.5	312 → 312	40	10			
JWH-C18	16.2	342 → 155	35	25			
COT-d3	5.6	180 → 80	25	18			
MOR-d3	6.6	289 → 289	45	5			
AP-d5	7.1	141 → 96	20	15			
MDMA-d5	7.7	199 → 165	20	15			
COC-d3	11.7	307 → 185	30	25			
BZE-d3	7.2	293 → 171	30	25			
THC-COOH-d3	14.8	348 → 330	30	16			

* In case of MOR, COD and 6-MAM, the protonated molecular ion was used for quantification.
[a] CV: cone voltage.
[b] CE: collision energy.

2.5. Method validation

Validation parameters included linearity, limits of detection (LOD) and quantification (LOQ), imprecision, inaccuracy, selectivity, carryover, matrix effect, recovery, process efficiency and stability studies. Linearity was determined by least-squares regression with $1/x^2$ weighting. Acceptable linearity was achieved when the coefficient of determination was at least 0.99 and the calibrators were quantified within ±20% at the LOQ and ±15% at other concentrations.

The LOD and LOQ were evaluated with decreasing analyte concentrations in drug-fortified breast milk. The LOD was defined as the lowest concentration with acceptable chromatography, the presence of all transitions with signal-to-noise ratios of at least 3, and a retention time within ±0.2 min of the average retention time of the calibrator. LOQ was the lowest concentration that met LOD criteria and a signal-to-noise ratio of at least 10.

Imprecision and inaccuracy were determined at three concentrations by analyzing five replicates on three different days ($n = 20$). Imprecision and inaccuracy, expressed as the coefficient of variation (%) of the measured values and error (%) respectively, were expected to be less than 20%.

Interferences from endogenous matrix components were evaluated by analyzing breast milk samples from ten healthy non drug-consuming volunteers fortified only with internal standards solutions. Endogenous interferences were considered insignificant if no peaks at LOQ value were detected at the retention times of the analytes in these ten breast milk samples. Potential interferences from other drugs of abuse, e.g. common benzodiazepines, and antidepressants were also evaluated by spiking 0.5 mL of prechecked drug-free human milk pool with 500 ng of each of the aforementioned substances (final concentration: 1000 ng/mL as the highest point of calibration curve) and carried through the entire procedure.

The potential for carryover was investigated by injecting extracted drug-free human milk, with added internal standards, immediately after analysis of the highest concentration point of the calibration curve and measuring the area of possible peaks at the retention times of the analytes under investigation. In case of CAF, carryover was also assessed after injecting three replicates two additional concentration points at 2000 and 5000 ng CAF per mL drug-free human milk, since this analyte was found in breast milk in concentration up to 4000 ng/mL [1,16]. These over-the-curve samples were also tested for calibration curve fitting, recovery, and imprecision once they were diluted 5-fold.

Matrix effects, recovery and process efficiency were determined using the experimental design proposed by Matuszewski et al. [32]. Set 1 were five replicates of QC material prepared in the mobile phase. Set 2 and 3 were five replicates of blank breast milk fortified with QC solutions after and before extraction, respectively. Matrix effects were determined by dividing mean peak areas of set 2 by set 1 multiplied by 100. A value of 100% indicates that the responses in the mobile phase and in the plasma extracts were the same and no matrix effect was observed. A value of >100% indicates an ionization enhancement and a value of <100% indicates an ionization suppression. Recovery was determined by comparing the mean peak areas of analytes under investigation obtained in set 3 to those in set 2 multiplied by 100. Process efficiency expressed as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of the same analyte standards (set 1) multiplied by 100 [32].

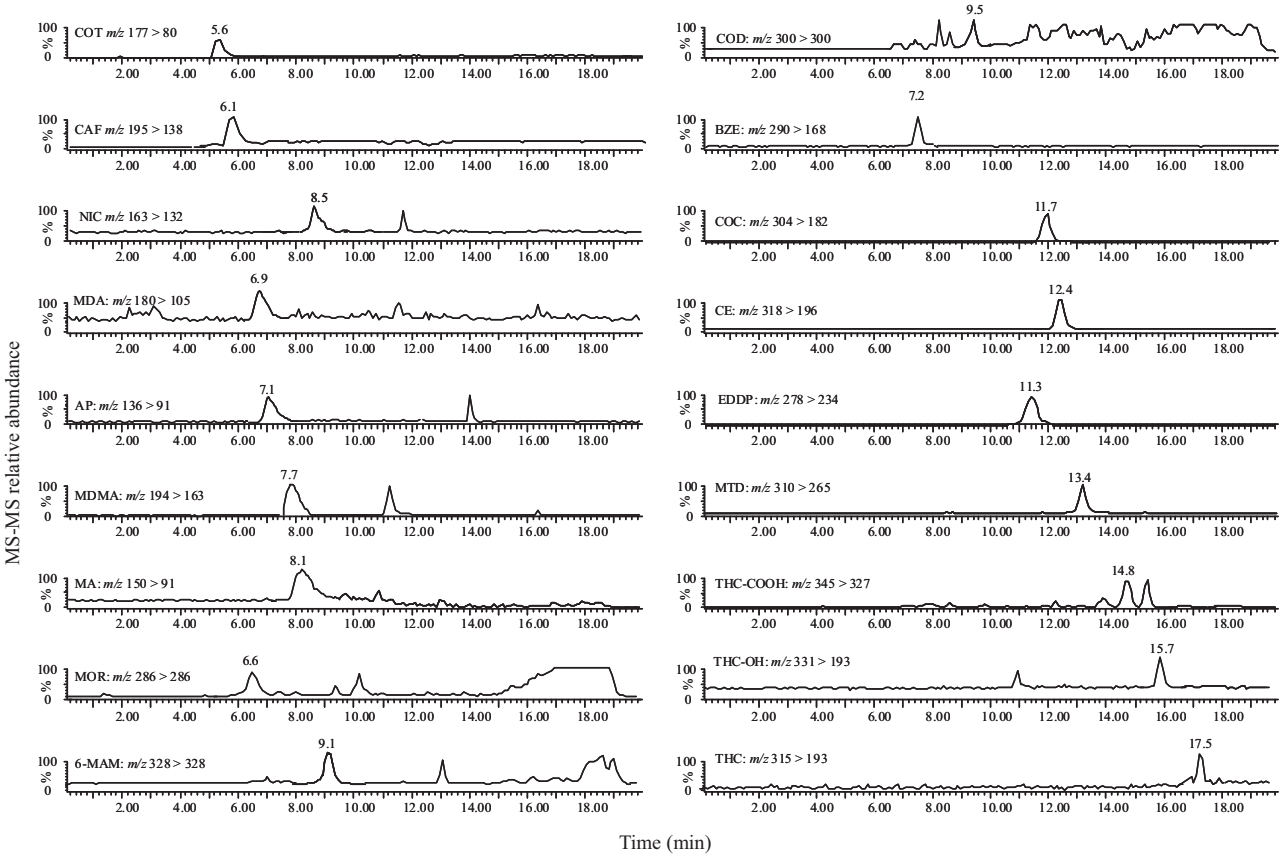

Fig. 1. LC–MS–MS chromatogram of a breast milk sample spiked at the low quality control concentration, 6 ng/mL for morphine (MOR), 6-acetylmorphine (6-MAM), codeine (COD), methamphetamine (MA), benzoylecgonine (BZE), cocaine (COC), Δ9-tetrahydrocannabinol (THC), 11-hydroxy-Δ9-tetrahydrocannabinol (TCH-OH), 11-nor-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) and 12 ng/mL for cotinine (COT), caffeine (CAF), nicotine (NIC), 3,4-methylenedioxyamphetamine (MDA), amphetamine (AP), 3,4-methylenedioxymethamphetamine (MDMA), cocaethylene (CE), methadone (MTD), 2-ethylene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP).

The effect of three freeze–thaw cycles (storage at −20 °C) on the compounds stability in human milk was evaluated by repeated analysis ($n = 3$) of QC samples. In addition, mid-term stability test was performed for real samples stored at −20 °C. Three replicates of four samples were analyzed once a month during a 6 months period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of the analytes both in QC and real samples. Finally, the effect of pasteurization on the stability of analytes under investigation in breast milk was evaluated by repeated analysis ($n = 3$) of QC samples. Milk was pasteurized following the method in use at the Spanish milk bank at the "Hospital Universitario 12 de Octubre" – Madrid Spain, by heating it at 62.5 °C for 30 min and then cooling it below 5 °C within 15 min. The pasteurization effect was expressed as a percentage of the initial concentration (first analyzed batch) of both analytes in QC samples.

All the validation parameters were calculated using two types of internal standards: the ones structurally related to the analytes under investigation and deuterated internal standards.

2.6. Breast milk samples

Breast milk samples came from the largest Spanish milk bank located at the "Hospital Universitario 12 de Octubre", Madrid, Spain. Samples were collected from January 2010 to June 2010. Once collected, milk from each mother was pasteurized as above reported, aliquoted and stored at −20 °C. At the time of donation, lactating mothers completed a structured questionnaire regarding smoking habits, consumption of caffeinated drinks and eventual consumption of psychoactive drugs. Mothers signed an informed consent to drug testing of donated milk and local ethics committee approved the protocol for milk donation and drug testing. Milk was accepted only from mothers declaring no toxic habit and no use of any drug or drug of abuse. However, no drug testing in any biological matrix was performed to confirm self-declarations. In order to check the eventual effects of pasteurization performed at the milk bank, 34 samples were sent in duplicate: before and after the pasteurization process.

Furthermore, to verify the reliability of developed method, breast milk samples from two addicted mothers declaring cocaine and cannabis consumption respectively and from one mother in methadone maintenance treatment were collected at Hospital del Mar, Barcelona, Spain.

3. Results

3.1. Chromatography and validation results

Representative chromatograms obtained following the extraction of drug-free milk spiked with all the analytes under investigation and real milk samples from addicted lactating mothers are shown in Figs. 1 and 2.

Linear calibration curves were obtained for the compounds of interest with correlation coefficients (r^2) of at least 0.99 in all cases and LODs and LOQs values were adequate for the purpose of the present study (Table 2). The intra and inter-assay imprecision (measured as coefficient of variation, CV) and inaccuracy (measured as % error) values were always lower than 20% (Table 3). Once diluted, over-the-curve CAF samples fitted the calibration curve, and when tested for imprecision and inaccuracy gave values always better

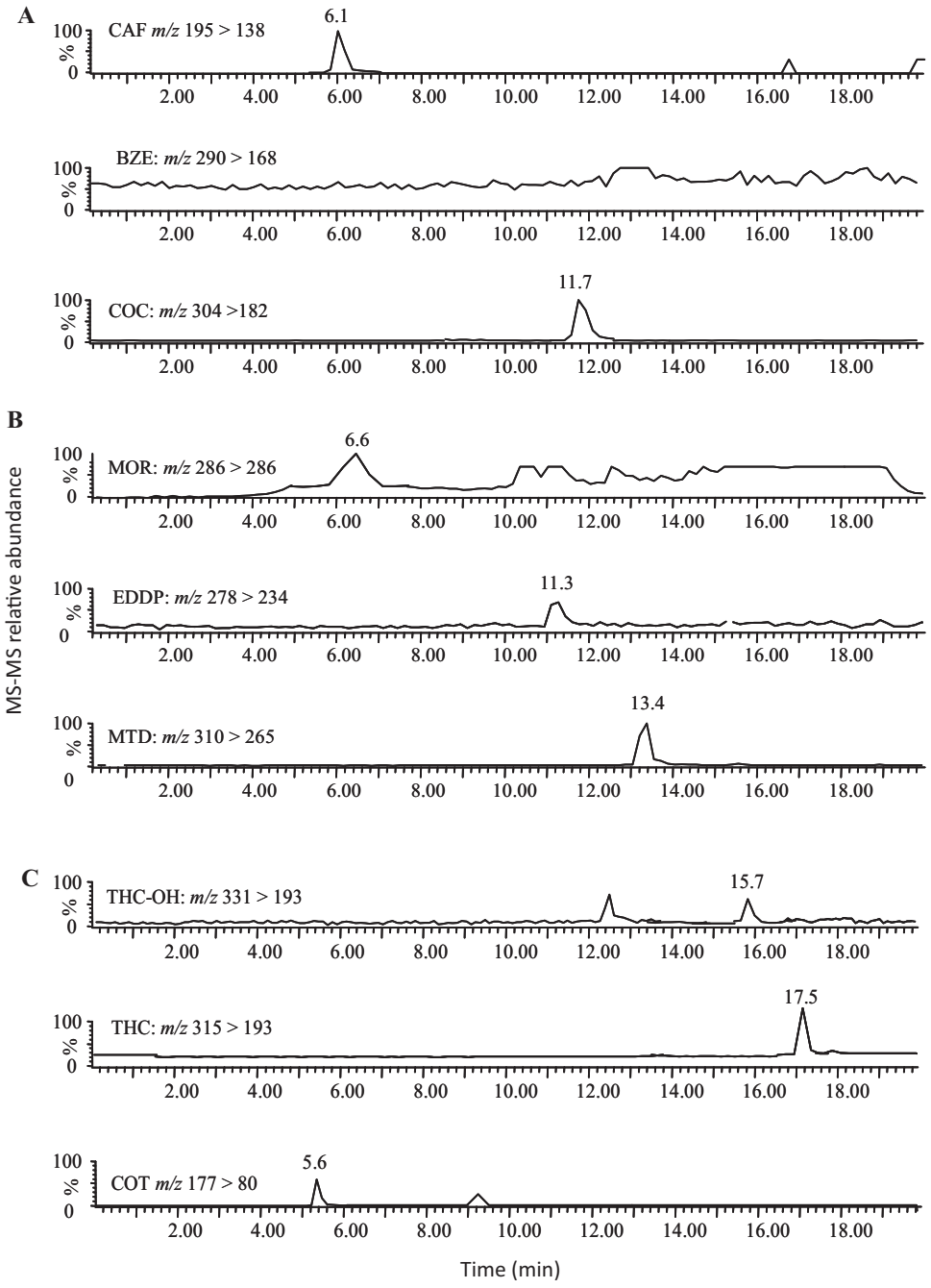

Fig. 2. LC–MS–MS chromatograms of breast milk samples from addicted nursing mothers containing: (A) cocaine (COC 5 ng/mL) and caffeine (CAF 539 ng/mL); (B) morphine (MOR 7 ng/mL), 2-ethylene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP 8 ng/mL) and methadone (MTD 97 ng/mL); (C) Δ-9-tetrahydrocannabinol (THC 86 ng/mL), 11-hydroxy-Δ-9-tetrahydrocannabinol (TCH-OH 5 ng/mL) and cotinine (COT 51 ng/mL).

than 20%. For this reason, breast milk samples with CAF concentration over the highest point of the calibration curve were diluted 1:5 and reanalyzed.

No additional peaks due to endogenous substances which could have interfered with the detection of the compounds of interest were observed in drug-free samples. No psychoactive drugs other than the compounds under investigation interfered with the assay. Blank samples injected after the highest point of the calibration curve or after 2000 and 5000 ng/mL CAF did not present any traces of carryover.

The mean absolute matrix effect ranged from 77.6% to 116.6%, recovery from 71.1% to 86.5%, and process efficiency from 46.8% to 84.0% (Table 4).

No relevant degradation was observed after any of the three freeze–thaw cycles, with differences in the initial concentration less than 10% for all the compounds under investigation. Similar results (differences to the initial concentration always lower than 10%) were obtained for real breast milk specimens with respect to the case of mid-term stability test, confirming the validity of stored samples for analysis. Similarly, no significant degradation was observed after pasteurization.

The above-reported validation parameters were obtained using internal standards structurally related to the analytes under investigation (NLP for opiates and COC, JWH-C18 for cannabinoids, MDPA for amphetamines and NENC for NIC, COT and CAF). Similar results were obtained when calculating the validation parameters

Table 2
Calibration results, limits of detection (LOD) and limits of quantification (LOQ).

Analyte	Slope ± SD	Intercept ± SD	Correlation coefficient (r^2) ± SD	LOD (ng/mL)	LOQ (ng/mL)
COT	0.028 ± 0.002	−0.226 ± 0.131	0.992 ± 0.001	2.0	7.0
CAF	0.002 ± 0.001	0.176 ± 0.036	0.992 ± 0.002	3.0	10.0
NIC	0.0008 ± 0.0002	0.036 ± 0.020	0.992 ± 0.002	3.0	10.0
MOR	0.0007 ± 0.0002	0.004 ± 0.006	0.995 ± 0.002	1.5	5.0
6-MAM	0.006 ± 0.001	−0.002 ± 0.013	0.994 ± 0.001	1.0	5.0
COD	0.005 ± 0.002	−0.005 ± 0.019	0.996 ± 0.004	1.0	5.0
MDA	0.0005 ± 0.0001	0.006 ± 0.006	0.993 ± 0.002	2.0	7.0
AP	0.0023 ± 0.0003	0.019 ± 0.007	0.994 ± 0.003	2.0	7.0
MDMA	0.003 ± 0.001	−0.022 ± 0.063	0.996 ± 0.005	2.5	8.0
MA	0.011 ± 0.002	0.100 ± 0.079	0.992 ± 0.001	1.0	5.0
BZE	0.0006 ± 0.0002	−0.005 ± 0.002	0.991 ± 0.001	1.0	5.0
COC	0.002 ± 0.001	0.036 ± 0.014	0.994 ± 0.003	1.0	5.0
CE	0.005 ± 0.001	0.103 ± 0.053	0.991 ± 0.001	2.0	7.0
MTD	0.010 ± 0.004	0.144 ± 0.075	0.991 ± 0.001	2.0	7.0
EDDP	0.004 ± 0.001	0.034 ± 0.061	0.994 ± 0.004	2.5	8.0
THC-COOH	0.002 ± 0.001	−0.026 ± 0.029	0.994 ± 0.004	1.0	5.0
THC-OH	0.0003 ± 0.0001	−0.005 ± 0.004	0.994 ± 0.003	1.5	5.0
THC	0.0001 ± 0.00002	0.002 ± 0.003	0.994 ± 0.004	1.5	5.0

using deuterated compounds (MOR-d3, COC-d3, BZE-d3, THC-COOH-d3, AP-d5, MDMA-d5 and COT-d3) as internal standards. For the analysis of real samples (more than 400 samples have already been analyzed and other 400 still to be analyzed), NLP, JWH-C18, MDPA and NENC were used because of the lower cost of the substances, higher stability and availability.

3.2. Analysis of breast milk samples

The method was first applied to breast milk specimens from the Spanish milk bank ($n = 400$). None of the analytes under investigation were found in the analyzed samples apart from caffeine, found in 17.5% ($n = 70$) of the breast milk specimens with a concentration ranging from 295 to 2191 ng/mL. These results are in accordance with previous studies [16–19], where CAF in breast milk from CAF-consuming mother was between 47 and 4000 ng/mL. CAF is considered compatible with breastfeeding [7] because occasional use appears to have little effects on infant but it would seem advisable to restrict caffeine consumption to less than 300 mg/day (approximately three cups of coffee) while breastfeeding [1]. With respect to the 34 milk samples collected and analyzed before and after pasteurization process, no difference in concentration of CAF, the only analyte found in few of these samples, was highlighted and none of the other psychoactive drugs were determined before or after the process.

Breast milk samples from two addicted mothers declaring cocaine and cannabis consumption respectively and from one mother in methadone maintenance treatment were collected at Hospital del Mar, Barcelona, Spain. COC was the only analyte found (concentration: 5 ng/mL) in the breast milk sample from the cocaine addicted mother, with BZE and other metabolites absent in this biological matrix and CAF present in high concentration (539 ng/mL). With respect to the cannabis smoker, THC (86 ng/mL) and THC-OH (5 ng/mL) were detected in her breast milk sample together with COT (51 ng/mL), the nicotine metabolite. Finally in breast milk from the heroin-addicted mother in methadone treatment, not only methadone (97 ng/mL) with its metabolite EDDP (8 ng/mL) were found but also a low concentration of MOR (7 ng/mL).

Table 3
Intra-day ($n = 5$) and inter-day ($n = 15$) precision and accuracy.

Analyte	Intra-day precision (RSD)			Intra-day accuracy (Error%)			Inter-day precision (CV%)			Inter-day accuracy (Error%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High	Low	Medium	High
COT	7.6	15.4	14.4	15.1	12.0	14.1	14.5	12.2	10.7	14.8	9.5	8.5
CAF	14.8	4.7	5.7	15.7	3.1	4.5	10.2	9.2	6.8	11.7	10.8	6.6
NIC	5.3	10.4	15.2	4.6	8.2	10.6	6.9	6.8	10.9	5.9	4.5	10.4
MOR	6.5	15.4	1.5	14.7	10.8	1.3	13.1	10.3	3.5	10.8	7.4	2.9
6-MAM	4.1	14.6	7.8	6.5	10.5	14.8	10.2	12.8	9.7	8.0	9.4	8.6
COD	10.6	5.3	2.4	12.4	5.07	2.7	9.6	11.8	8.5	9.4	10.7	6.4
MDA	7.4	8.9	1.7	6.6	9.8	1.6	9.4	10.5	9.5	7.2	13.5	5.9
AP	9.5	6.8	3.4	6.5	4.4	7.6	9.8	9.3	7.3	7.4	6.5	6.4
MDMA	11.8	12.0	12.3	11.9	8.8	8.6	11.5	10.3	10.6	10.2	10.3	6.2
MA	10.0	11.1	5.1	8.2	10.1	4.3	12.4	11.1	4.9	11.8	10.9	4.3
BZE	6.2	5.6	2.5	9.9	8.0	2.3	8.7	10.6	4.3	10.0	7.6	2.9
COC	1.5	6.5	1.6	10.1	7.4	1.7	10.2	10.4	5.2	8.7	7.8	3.4
CE	3.0	10.3	6.5	15.7	11.6	6.8	10.6	10.5	9.9	10.8	10.9	8.9
MTD	3.5	10.0	7.4	2.2	6.9	13.5	10.1	9.1	7.4	6.7	7.3	8.2
EDDP	5.3	9.1	11.3	4.5	11.8	7.3	9.4	10.8	7.4	8.6	10.9	5.9
THC-COOH	2.9	9.2	9.0	14.9	11.5	8.9	10.6	7.8	8.2	11.5	8.4	5.3
THC-OH	1.1	4.6	0.6	10.8	6.2	2.6	10.4	8.6	2.6	9.5	7.6	1.9
THC	9.2	5.1	13.6	7.1	4.3	10.2	9.9	5.0	9.2	7.6	5.1	6.9

Table 4
Matrix effect, recovery and process efficiency data for analytes under investigation in five different lots of human breast milk.

Analyte	Matrix effect (%)			Recovery (%)			Process efficiency (%)		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
COT	98.1	94.4	95.1	72.9	72.0	77.4	71.5	68.0	73.6
CAF	104.4	105.4	104.9	61.4	56.2	59.6	64.1	59.2	62.5
NIC	90.0	96.0	91.9	52.0	55.8	54.6	46.8	53.6	50.2
MOR	95.1	96.1	100.2	66.9	70.7	63.5	63.7	67.9	63.6
6-MAM	90.5	90.8	90.6	82.9	83.6	84.0	75.0	75.9	76.1
COD	95.0	94.9	91.1	77.9	83.2	83.2	74.0	79.0	75.8
MDA	88.0	82.8	85.7	68.3	70.4	64.1	60.1	58.3	54.9
AP	103.2	102.6	105.0	56.7	54.0	51.6	58.5	55.4	54.2
MDMA	94.7	97.1	98.0	72.6	71.5	69.0	68.8	69.4	67.6
MA	94.5	92.5	95.8	67.9	70.8	65.3	64.2	65.5	62.6
BZE	94.0	92.2	87.4	62.0	61.9	66.4	58.3	57.1	58.0
COC	93.8	93.2	95.2	81.9	83.0	75.3	76.8	77.4	72.1
CE	85.1	88.0	87.6	86.5	82.4	82.4	73.6	72.5	72.2
MTD	77.6	71.1	75.6	73.9	85.4	72.8	57.4	60.7	55.0
EDDP	114.8	116.6	113.0	65.5	64.2	64.1	75.2	74.8	72.4
THC-COOH	92.1	94.1	92.8	56.0	53.2	55.6	51.6	50.1	51.6
THC-OH	104.8	105.1	102.6	57.8	59.9	59.3	60.6	63.0	60.8
THC	88.0	91.8	91.0	59.9	59.8	63.5	52.7	54.9	57.8

4. Discussion

There is ample evidence in the literature that breast feeding is beneficial in meeting the nutritional and immunological needs of all babies, whether born at full-term or prematurely. Ideally, the milk should come from the baby's mother, but sometimes this is impossible. Mothers of preterm babies and other babies in intensive care are often unable, too sick or simply they do not have sufficient production in the first day after delivery to provide enough milk for their baby's needs. In these cases the milk from the human milk bank can be a viable alternative. In fact, with respect to the infections transmission, there are three different filters to check the milk from the bank: first a structured interview to the donating mothers on life style, second a complete blood analysis and third the milk pasteurization, which removes potentially harmful viruses and bacteria.

However, due to the large prevalence of drug use in the population of child bearing age, it is feasible that the milk donated by mothers could potentially contain drugs which may be harmful to the infants. Screening the donated milk for the presence of drugs of abuse prior to being given to the newborns is extremely important. The method we describe has the advantage that it quantifies simultaneously a large number of potentially harmful licit and illicit drugs.

With respect to the milk sample collected from the cocaine addicted mother, considering that COC is readily soluble in non polar solvents, its distribution into lipid-rich breast milk is predictable. More polar COC metabolites, such as BZE, may be more soluble in blood, and this could be the cause of disproportionate partitioning of COC relative to its metabolite, in breast milk [22]. Furthermore, it is likely that the concentration of COC and BZE in breast milk is a function of the temporal relationship between COC use and collection of specimen. Breastfeeding while consuming COC is absolutely not safe in the light of evidence that COC concentrations in breast milk reaches high levels if the lactating mothers regularly use COC. Milk samples collected from a mother declaring cannabis smoking showed the presence of THC and THC-OH together with NIC and COT confirming that cannabis was consumed with tobacco and that THC-COOH, the acid THC metabolite was not present in this sample. Finally, the presence of low concentrations of morphine was identified together with methadone and its metabolite in the milk sample from the mother on methadone maintenance program demonstrating a possible relapse in opiates consumption.

5. Conclusion

For the first time a fully validated LC–MS/MS method simultaneously quantifies the most frequently used licit and illicit psychoactive drugs in human breast milk. The method involved a fast and simple sample extraction procedure, presented a high throughput, adequate linearity, accuracy and precision to be used for rapid screening of milk samples continuously arriving at a milk bank.

Breastfeeding mothers are often reluctant to admit to using drugs or they may not even be aware that they are using a drug so measurement of drug concentrations (licit or illicit drugs) in breast milk provides useful information for appropriate maternal counselling, immediate infant treatment and subsequent medical follow-up.

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