Development and Validation of an EI-GC–MS Method for the Determination of Methadone and its Major Metabolites (EDDP and EMDP) in Human Breast Milk

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Abstract

Methadone is used extensively for the maintenance of opioid-addicted pregnant women. Because methadone and the two major metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP), are excreted into breast milk, a sensitive and specific gas chromatographic–mass spectrometric method has been developed, optimized, and validated for their quantitative determination in human breast milk. The procedure combined protein precipitation with acetonitrile and solid-phase extraction, using Isolute Confirm HCX mixed-mode SPE columns, with minimal matrix effect. The optimum extraction conditions for all three analytes were evaluated using spiked human breast milk, and the recovery exceeded 93.0%. This assay uses methadone-d9 as internal standard for the determination of methadone and EMDP, and EDDP-d3 for the determination of EDDP. Calibration curves were linear within the range of 2.00–1000 µg/L for methadone ($R^2 > 0.995$) and 1.00–500 µg/L for EDDP ($R^2 > 0.997$) and EMDP ($R^2 > 0.991$). Intra- and interday accuracy and precision were within the range of 0.8–5.7% and 1.3–5.2%, respectively, for all analytes. The stability study was assessed by fortifying human breast milk with methadone and its metabolites at two different concentrations and keeping the samples at different temperature conditions. The analytes were found to be stable in breast milk at room temperature for at least 4 h and at –20°C for at least one month. The method was used for the determination of methadone and its major metabolites in human breast milk samples obtained from women in the postpartum period participating in a methadone maintenance program.

Introduction

Methadone is widely used for opioid substitution treatment, and has been accepted as a therapeutic strategy for the treatment of opioid addicts during pregnancy since the early 1970s (1). It is also the only maintenance treatment approved in Greece for opiate-addicted women during pregnancy and in the postpartum period (2). Methadone is metabolized by demethylation to two main metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) (3). Methadone is a lipophilic, weakly basic (pKₐ 8.25) compound that is highly protein bound, and it is excreted into breast milk (4,5). The peak methadone levels in milk occur at approximately 4 h after an oral dose, or 1 to 2 h after the peak plasma levels (6).

Pregnant women in methadone maintenance programs frequently express a desire to nurse their babies but they are discouraged from doing so, due to the concern of the amount of methadone that will be passed to the newborns by breastfeeding (7). Post-feed breast milk methadone concentrations are approximately 33% higher than pre-feed concentrations because of the increase in lipid content of breast milk that occurs during the course of a feeding (5).

The prenatal exposure to methadone could result in neonates with a variety of neurobehavioral consequences (8). Methadone-exposed newborns develop neonatal abstinence syndrome (NAS) at 60–90% and present a number of symptoms frequently requiring prolonged hospitalization and treatment. The symptomatology of an infant undergoing mild or acute NAS includes hypertonicity, irritability, abnormal movements, sleeping disorders, hypersensitivity, and feeding problems (2,9). Pharmacologic therapy for NAS is based on the administration of methadone, morphine, or phenobarbitone (3,5–7,9,10). Breastfeeding has been suggested as an alternative or supplementary treatment for managing the symptoms of withdrawal in methadone-exposed infants (3,9–11). Nevertheless, there is limited knowledge concerning the safety or therapeutic benefits of breastfeeding, and knowing the concentration of methadone in breast milk is critical for predicting possible toxicological consequences to the infant.

Gas chromatography (GC) (6,7,12–26) or liquid chromatography (3–5,27–29) with mass spectrometry (MS)
A centrifuge (ALRESA, Spain) at a speed of 2000 rpm. Evaporation of all samples. Centrifugation was performed with Vap PIERCE Model 18780, Rockford, IL) was used for the electrode. An evaporating unit connected with nitrogen (Reacti- use was a METROHM product with a glass combination electrode. The digital pH-meter used for the mixing of samples and standards. The digital pH-meter is essential for calculating the amount of methadone and metabolites to which the infant would be exposed during breastfeeding (6).

The purpose of our work was to develop, optimize, and validate a sensitive and accurate GC–MS method for the determination of methadone and the major metabolites, EDDP and EMDP, in breast milk. The work was undertaken in order to quantify the distribution and excretion of methadone and metabolites into human breast milk and investigate the exposure of breastfed infants to the drug. The method includes protein precipitation with acetonitrile combined with SPE as an efficient isolation procedure. This assay can be a useful tool for the investigation of the transfer of methadone and its metabolites from the breast milk of methadone-maintained women to their infants. It can also be used for judging the effectiveness of breastfeeding in the “treatment” of newborns with NAS, or for avoiding toxic consequences for the newborn if methadone breast milk levels are extremely high.

**Experimental**

**Apparatus**

The analysis was performed on a Shimadzu GC–MS model QP 2010S, equipped with a DB-5MS column (30 m × 0.25-mm i.d., 0.25-µm film thickness), and helium was used as carrier gas. Injections of 1 µL were carried out in the splitless mode using a Shimadzu AOC-20i autosampler system. The MS was operated in electron impact (EI) ionization and selective ion monitoring (SIM) mode for the quantitation of methadone and metabolites EDDP and EMDP.

In this study, the SPE columns Isolute Confirm HCX were used. A vortex (Chiltern model MT 19), set at speed 5, was used for the mixing of samples and standards. The digital pH-meter used was a METROHM product with a glass combination electrode. An evaporating unit connected with nitrogen (Reacti-Vap PIERCE Model 18780, Rockford, IL) was used for the evaporation of all samples. Centrifugation was performed with a centrifuge (ALRESA, Spain) at a speed of 2000 rpm.

Chemicals and reagents

Methadone, EDDP, EMTP, methadone-d₉, and EDDP-d₃ were purchased from LGC Promochem (Molsheim, France). All standards were > 99.9% pure, as described by the manufacturer. HPLC-grade methanol, acetonitrile, and dichloromethane were obtained from Merck (Darmstadt, Germany). Analytical-grade ammonium hydroxide (NH₄OH), glacial acetic acid (CH₃COOH), and extra pure sodium-dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O) were obtained from Merck.

Phosphate buffer (pH 6.00, 0.1 M) was prepared by dissolving the appropriate amount of NaH₂PO₄·2H₂O in water and adjusting the pH to 6.00 with the addition of NaOH (0.1 N) using the pH-meter.

Pooled, drug-free breast milk was obtained from local, healthy, breastfeeding female volunteers (n = 6) and verified as negative for drugs by GC–MS.

**Calibration standards, internal standards, and quality control samples**

Stock standard solutions (100 mg/L) of analytes were prepared in methanol for methadone and EDDP and in acetonitrile for EMDP. Stock standard solutions (100 mg/L) of methadone-d₉ and EDDP-d₃ were prepared in methanol.

Eight aqueous combined working standard solutions containing 0.04–20 mg/L methadone and 0.02–10 mg/L EDDP and EMDP were prepared by mixing the appropriate volumes of the corresponding stock solutions of each compound and dilution with water. Spiked breast milk standards for calibration curves (calibrators) were constructed by spiking 1.00 mL aliquots of blank breast milk with 50 µL of the combined working standard solutions. The eight calibrators contained methadone at 2.00, 4.00, 10.0, 40.0, 100, 200, 500, and 1000 µg/L and EDDP and EMDP at 1.00, 2.00, 5.00, 20.0, 50.0, 100, 250, and 500 µg/L.

Additional aqueous combined working standard solutions (in three different concentrations) containing methadone, EDDP, and EMDP were prepared from different stock solutions from what was used for calibrators, in order to prepare breast milk quality control (QC) samples. The three breast milk QC samples contained methadone at 6.00, 400, and 800 µg/L, as well as EDDP and EMDP at 3.00, 200, and 400 µg/L, and were prepared in a similar way as that of the calibrators. Fresh working solutions were prepared on a daily basis.

A combined aqueous working internal standard solution containing methadone-d₉ at 4.00 mg/L and EDDP-d₃ at 2.00 mg/L was prepared by mixing the appropriate volumes of the corresponding methanolic stock standard solutions and dilution with water. This assay uses methadone-d₉ as internal standard for the determination of methadone and EMDP, and EDDP-d₃ for the determination of EDDP.

Calibration curves, based on the peak-area ratio of the analyte to the corresponding internal standard against analyte concentration, were constructed daily and used for the calculation of the analyte concentration in the QC samples and in the patient breast milk samples.

**GC–MS analysis**

The developed GC method was optimized for column temperature program, flow rate of carrier gas and temperatures of injector, ion source, and interface. The finally optimized GC conditions were as follows: flow rate of carrier gas 1.30 mL/min, initial column temperature 80°C increased at a rate of 40°C/min to the intermediate step temperature of 270°C.
with 2 min hold, followed by an increase at a rate of 40°C/min to the final column temperature of 300°C and held for 4 min. Injector, ion source, and interface temperatures were maintained at 260°C, 200°C, and 280°C, respectively. Methadone, EDDP, EMTP, methadone-d9, and EDDP-d3 were quantified at the base peaks of m/z 72, 277, 208, 78, and 280, respectively.

**Extraction procedure**

To 1.00 mL of breast milk (calibrator, QC sample, or patient sample) 50 µL of the combined working internal standard solution were added and vortex mixed for 15 s. Hence, all samples used contained 200 and 100 µg/L methadone-d9 and EDDP-d3, respectively. The samples were then diluted with 1.00 mL of deionized water, 3.00 mL of acetonitrile were added dropwise while vortex mixing, for the protein precipitation, and were centrifuged at 2000 rpm for 5 min. The organic supernatant phase was decanted into a clean glass tube and the solvent was evaporated under a gentle stream of N2 at 40°C to approximately 1.5 mL. The pH of samples was adjusted to 6.00 with the addition of 3.00 mL of phosphate buffer 0.1 M (pH 6.00).

The SPE columns were conditioned with 3 mL of methanol, 3 mL of deionized water, and 1 mL of phosphate buffer 0.1 M (pH 6.00) prior to sample loading. The samples were applied to the columns at a flow rate of approximately 1.0 mL/min. Consequently, the columns were washed successively with 3 mL of deionized water, 1 mL of 1 M acetic acid, and 3 mL of methanol, and then they were dried under maximum vacuum for 5 min. The analytes were eluted twice with 1.5 mL of freshly prepared mixture of dichloromethane/methanol/ammonium hydroxide (90:10:2, v/v/v). The eluates were collected in silanized tubes and evaporated to dryness under a gentle stream of N2 at 40°C. The residue was reconstituted with 60 µL of acetonitrile, and a 1-µL aliquot of the resulting solution was injected onto the GC–MS system.

**Method validation**

The following criteria were used to evaluate the GC–MS method: selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, precision, accuracy, recovery, interference from exogenous compounds, robustness, and stability. Method validation was accomplished through six analytical runs within six different days.

**Results and Discussion**

Breast milk is a complex biological fluid with high protein and fat content that requires sample pretreatment to remove endogenous materials, like proteins and lipids, while its composition changes during the postpartum and feeding period. The present work describes an analytical procedure for the simultaneous determination of methadone and its metabolites, EDDP and EMTP, in human breast milk by GC–MS with EI ionization, following protein precipitation and SPE, and it is the first validated GC–MS method for the determination of methadone, EDDP, and EMTP in human breast milk.

During preliminary studies, a plethora of organic solvents were investigated for liquid–liquid extraction of breast milk but extended matrix effect interfered severely with identification of analytes. Back extraction (3,5,6) was also examined but led to poor recovery for all analytes. Subsequently, when breast milk was directly applied to different types of SPE columns (7), the flow rate became irregular because of the high lipid and protein content of the matrix. An LC–MS–MS method has been published recently (4) based on protein precipitation with methanol before application of breast milk to SPE columns. Hence, it was decided to investigate the combination of protein precipitation with acetonitrile and SPE, using Isolute HCX SPE columns, in order to eliminate matrix effects and increase extraction efficiency. The procedure chosen was successful and was further investigated.

Representative chromatograms obtained from extracted blank and spiked breast milk samples (methadone: 6.00 µg/L, EDDP and EMTP: 3.00 µg/L) are shown in Figures 1 and 2, respectively. A chromatogram obtained from a representative breast milk specimen collected from a mother maintained on 30 mg methadone/day is shown in Figure 3.

Separation of the analytes of interest and their respective internal standards was achieved within 6 min, and the total
chromatographic run time was under 12 min. The retention times of methadone, EDDP, and EMDP were 5.98, 5.60, and 5.30 min, respectively.

In order to investigate, in this study, whether analytes need some time to equilibrate with milk after spiking, we analyzed freshly spiked milk samples and milk samples allowed to equilibrate with analytes after spiking for 24 h. In both cases, we received the same results.

Selectivity was established by analyzing six blank samples from different origins and the matrix effect of endogenous compounds was evaluated as minimal, because no co-eluting endogenous compounds that might interfere with the accurate determination of the analytes of interest were observed.

Extraction efficiency for QC samples was assessed with five replicates at the low and high concentrations and with eight replicates at the intermediate concentration. Absolute recovery for each analyte at three concentration levels was calculated as the percentage of each analyte response at the sample compared to that of a solution containing the analyte at the corresponding concentrations and was found to be higher than 93.0% (Table I). Furthermore, absolute recoveries for 200 µg/L methadone-d₉ and 100 µg/L EDDP-d₃ were found equal to 95.5% (± 3.4, n = 8) and 92.9% (± 6.7, n = 8), respectively.

Linearity was investigated by calculation of the linear regression equation by the method of least-squares with a weighting factor of 1/x² and expressed by the correlation coefficient (R²). Each calibrator was back calculated against the full curve. Results show that linearity extended within the range 2.00–1000 µg/L for methadone and 1.00–500 µg/L for EDDP and EMDP, with correlation coefficients > 0.991%. LOD and LOQ for each analyte were calculated (n = 6), taking into account a signal-to-noise ratio of at least 3:1 and 10:1, respectively. Mean linear regression equations of the analytes, interday precision of slopes (% RSD), linearity, LOD, and LOQ are presented in Table II.

Precision and accuracy of the method were evaluated by analyzing three QC samples at concentrations within the linear range of each analyte (6.00, 400, and 800 µg/L for methadone; 3.00, 200, and 400 µg/L for EDDP and EMDP). Precision (intraday n = 6 and interday n = 36) was expressed as the relative standard deviation (% RSD). Accuracy of the method was calculated as the percent difference from the expected concentration (% Eᵣ). Results (Table III) show that precision and accuracy were less than 5.2% and 5.7% for all analytes at all concentrations.

![Figure 2. Representative SIM chromatograms of a low QC breast milk sample (methadone: 6.00 µg/L, EDDP and EMDP: 3.00 µg/L).](image1)

![Figure 3. Representative SIM chromatograms of a breast milk specimen collected on the second day of the postpartum period from a mother maintained on methadone at a dose of 30 mg/day; calculated concentration of methadone (83.7 µg/L), EDDP (18.8 µg/L), and EMDP (1.67 µg/L).](image2)
Interference from exogenous compounds was investigated for substances that were often used by participants in methadone maintenance programs. A methanolic standard mixture solution of commonly used illicit and licit drugs or their metabolites (morphine, codeine, 6-acetylmorphine, Δ9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, cocaine, ecgonine methylester, benzoyloecgonine, diazepam, nordiazepam, bromazepam, alprazolam, 7-aminoflunitrazepam, phenobarbitone, amitriptyline, clomipramine, amphetamine, methamphetamine, MDMA, ephedrine, and ketamine) at 100 mg/L was injected onto the GC–MS system. No interference of these substances was observed at the retention time of the analytes of interest. Additionally, spiked breast milk samples (n = 6) containing methadone, EDDP, and EMDP at low QC levels and the previously mentioned substances at 1 mg/L were also analyzed according to our method. Our study documented that the previously mentioned compounds did not interfere with the accurate determination of methadone, EDDP, and EMDP in human breast milk.

Robustness of the entire method was studied by slightly altering experimental parameters on extraction procedure and chromatographic separation. No effect was found for all analytes studied by using pH 6.50 instead of 6.00, a different lot number of SPE columns, and a different ratio of SPE elution solvents, as well as flow rate of carrier gas at 1.26 mL/min instead of 1.30 mL/min, injector temperature 257°C instead of 260°C, intermediate step column temperature 273°C instead of 270°C, and 3% lower detector voltage.

Stability study was assessed by analyzing fortified breast milk with 9.00 and 700 µg/L methadone and 4.50 and 350 µg/L for EDDP and EMDP and keeping the samples at room temperature and at –20°C. Furthermore, fortified breast milk specimens were subjected to three freeze-thaw cycles. The overall maximum loss of the analytes of interest, at two concentrations (low and high), under these experimental conditions was not more than 3.9% (Table IV). Methadone and metabolites in human breast milk were found to be stable at room temperature for at least 4 h and at least one month at –20°C.

### Table I. Percent Absolute Extraction Recoveries of Methadone, EDDP, and EMDP at Three QC Concentrations

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration</th>
<th>Mean Absolute Recovery ± SD</th>
</tr>
</thead>
</table>
| Methadone | 6.00 µg/L (n = 5) | 97.4 ± 4.6  
400 µg/L (n = 8) | 94.7 ± 3.7  
800 µg/L (n = 5) | 97.8 ± 4.6 |
| EDDP | 3.00 µg/L (n = 5) | 95.0 ± 2.2  
200 µg/L (n = 8) | 93.1 ± 6.2  
400 µg/L (n = 5) | 96.8 ± 5.0 |
| EMDP | 3.00 µg/L (n = 5) | 95.9 ± 3.6  
200 µg/L (n = 8) | 93.0 ± 2.9  
400 µg/L (n = 5) | 98.6 ± 3.9 |

### Table II. Mean Linear Regression Equations, Interday Precision of Slopes*, Linearity, Limits of Detection† and Quantification‡ for Methadone, EDDP, and EMDP in Breast Milk

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration Range (µg/L)</th>
<th>Mean Linear Regression Equations</th>
<th>% RSD of Slopes (n = 6)</th>
<th>R²</th>
<th>LOD (µg/L)</th>
<th>LOQ (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methadone</td>
<td>2.00–1000</td>
<td>y = 0.00525 (± 0.00011)C + 0.00557 (± 0.00055)</td>
<td>2.1</td>
<td>&gt; 0.995</td>
<td>0.6</td>
<td>2.0</td>
</tr>
<tr>
<td>EDDP</td>
<td>1.00–500</td>
<td>y = 0.01028 (± 0.00043)C + 0.0103 (± 0.0021)</td>
<td>4.2</td>
<td>&gt; 0.997</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>EMDP</td>
<td>1.00–500</td>
<td>y = 0.000689 (± 0.000038)C + 0.00074 (± 0.00015)</td>
<td>5.5</td>
<td>&gt; 0.991</td>
<td>0.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* % RSD.
† LOD, n = 6.
‡ LOQ, n = 6.
§ y = peak-area ratio of analyte/corresponding internal standard.

### Table III. Intraday and Interday Accuracy and Precision of Methadone, EDDP, and EMDP from Breast Milk QC Samples

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration Added (µg/L)</th>
<th>Concentration found mean ± SD (µg/L)</th>
<th>Accuracy (% Er)</th>
<th>Precision (% RSD)</th>
<th>Concentration found mean ± SD (µg/L)</th>
<th>Accuracy (% Er)</th>
<th>Precision (% RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methadone</td>
<td>6.00</td>
<td>6.18 ± 0.16</td>
<td>3.0</td>
<td>2.6</td>
<td>6.16 ± 0.16</td>
<td>2.6</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>388 ± 10</td>
<td>–3.0</td>
<td>2.6</td>
<td>395 ± 11</td>
<td>–1.3</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>787 ± 11</td>
<td>–1.6</td>
<td>1.4</td>
<td>788 ± 14</td>
<td>–1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>EDDP</td>
<td>3.00</td>
<td>3.03 ± 0.09</td>
<td>0.9</td>
<td>3.1</td>
<td>3.02 ± 0.09</td>
<td>0.8</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>188.6 ± 2.4</td>
<td>–5.7</td>
<td>1.3</td>
<td>196.5 ± 7.2</td>
<td>–1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>EMDP</td>
<td>3.00</td>
<td>383.2 ± 7.6</td>
<td>–4.2</td>
<td>2.0</td>
<td>397 ± 12</td>
<td>–0.8</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>202.8 ± 3.5</td>
<td>1.4</td>
<td>1.7</td>
<td>304 ± 0.12</td>
<td>1.3</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>414 ± 19</td>
<td>3.6</td>
<td>4.6</td>
<td>403 ± 21</td>
<td>0.7</td>
<td>5.2</td>
</tr>
</tbody>
</table>
Method application

The method was applied to breast milk specimens collected from three women who were methadone-maintained for at least the last month of their pregnancy. The women were receiving a single daily dose of 70 and 40 mg, or a divided daily dose of 30 mg methadone. The samples were collected by breast pump before and 4 h after oral methadone intake for two days of the postpartum period and the total volume of breast milk was pumped out each time. Breastfeeding of newborns from methadone-maintained mothers did not take place in this study because it is not yet allowed in Greece.

From the results obtained (Table V), it can be concluded that methadone milk levels in the three cases studied were found to be increasing 4 h after methadone administration during the first and the second day of the study. Moreover, on both days, all EMDP milk levels were lower than those of EDDP. Methadone concentrations in milk were found higher during the second day in comparison with the corresponding concentrations during the first day of study. In most cases, similar results were observed for EDDP and EMDP.

Conclusions

This paper describes a GC–MS method for the simultaneous determination of methadone, EDDP, and EMDP in human breast milk. The use of protein precipitation and SPE procedure provides minimal matrix effect and no interference from commonly used drugs. In comparison with previously reported GC–MS methods (6,7) or HPLC and LC–MS–MS methods (2–5) concerning the determination of methadone and its metabolites in breast milk, the present methodology shows improved sensitivity, wider linearity range, enhanced accuracy and precise data, higher extraction recoveries, and is robust in a combination of minor variations in the procedure or in the chromatographic conditions. To our knowledge, this is the only published GC–MS method that simultaneously determines methadone and its two metabolites in human breast milk and provides full validation data. The only previously reported fully validated similar method (4) included protein precipitation with methanol and SPE prior to LC–MS–MS analysis, whereas in our method, we used a combination of protein precipitation with ACN and SPE prior to GC–MS analysis. It has to be mentioned here that GC combined with MS is the most common analytical technique used by toxicological laboratories and is available worldwide.

Application of the method to human breast milk samples collected from mothers participating in methadone maintenance programs achieved accurate determination of total concentrations of methadone and its metabolites during the postpartum period. Subsequently, this method could provide useful data for critical decision making concerning breastfeeding permission, may be of great use in solving of clinical and forensic issues, and could be applied in different pharmacokinetic studies.

Acknowledgments

The authors gratefully acknowledge the mothers who participate in our study and the Greek Organization Against Drugs for making this study possible. This research was financially supported by Special Research Account of the University of Athens.

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| Table IV. Stability of Methadone and Metabolites in Human Breast Milk, Calculated as Percent Loss for Each Analyte, at Two Concentration Levels (Low and High) for Certain Parameters |
|-------------------|------------|------------|------------|------------|
| Analyte | Methadone | EDDP | EMDP |
| Concentration (µg/L) | 9.00 | 700 | 4.50 | 350 | 4.50 | 350 |
| 25°C, 4 h | –1.6 | –0.1 | –0.4 | –0.1 | –1.2 | –0.7 |
| –20°C, 30 days | –1.9 | –1.7 | –1.8 | –0.7 | –3.2 | –1.9 |
| Three freeze-thaw cycles | –1.1 | –0.5 | –1.2 | –0.4 | –3.9 | –3.5 |

| Table V. Concentrations (µg/L) of Methadone, EDDP, and EMDP in Breast Milk of Three Cases, Before and 4 h After Oral Administration of Methadone |
|-------------------|------------|------------|------------|------------|
| Dose | Case 1 | Case 2 | Case 3 |
| | 70 mg/day | 40 mg/day | 30 mg/day |
| | Before | 4 h After | Before | 4 h After | Before | 4 h After |
| 1st day | Methadone (µg/L) | 88.5 | 99.0 | 32.0 | 104.5 | 58.7 | 83.7 |
| | EDDP (µg/L) | 6.31 | 6.18 | 5.15 | 14.0 | 13.8 | 18.8 |
| | EMDP (µg/L) | 1.30 | 2.61 | * | * | 1.09 | 1.67 |
| 2nd day | Methadone (µg/L) | 96.6 | 146.0 | 97.4 | 144.7 | 77.3 | 93.2 |
| | EDDP (µg/L) | 7.91 | 3.62 | 16.2 | 18.1 | 20.5 | 19.6 |
| | EMDP (µg/L) | 1.25 | 3.17 | 1.25 | 1.62 | 2.57 | 2.53 |

* Concentration above LOD and below LOQ.


7. S. Paterson, R. Cordero, and S. Burlinson. Screening and semi-


18. S. Paterson, R. Cordero, and S. Burlinson. Screening and semi-


