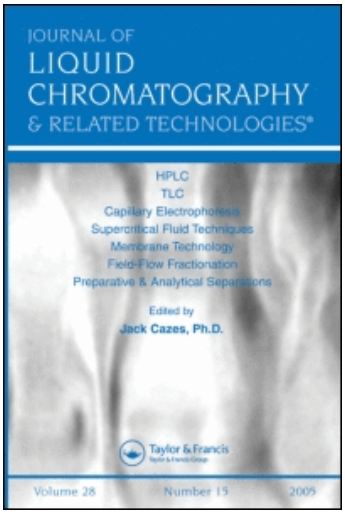


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# SOLID-PHASE EXTRACTION WITH HPLC-DAD SELECTIVE DETECTION OF AMITRIPTYLINE AND NORTRIPTYLINE IN HUMAN BREAST MILK

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## ABSTRACT

A simple, rapid, highly selective and sensitive method was developed to determine concentrations of amitriptyline and nortriptyline in human breast milk, using high performance liquid chromatography (HPLC) with a diode-array detector (DAD). Drugs were effectively extracted from 250 μL of milk by a solid phase extraction procedure on a polymeric sorbent-C18-based cartridge. The separation was carried out on a Eclipse XDB-C18 reversed phase column (30 mm × 4.6 mm, 3.5 μm particle size) using the mobile phase composed of acetonitrile and ammonium acetate 0.1M (33/67, v/v). Imipramine was used as internal standard. Calibration functions were linear in the calibration range of 60-300 ng.mL$^{-1}$. The lower limit of detection was 1.9 ng.mL$^{-1}$ for both compounds. The recoveries were 92% for amitriptyline and

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89% for nortriptyline. Since no method have been reported in the literature for the dosage of these two drugs in breast milk, this method could be useful, particularly, when evaluating the potential risk for the neonate during the lactation period.

## INTRODUCTION

The postpartum period is a time of great physical and emotional changes. The incidence of psychiatric illness is potentially higher in this period and may require an antidepressant therapy. Amitriptyline is an effective tricyclic antidepressant used for treatment of depressive disorders during pregnancy and lactation period. Amitriptyline undergoes an extensive metabolism to form mainly nortriptyline, which is the active metabolite responsible for the clinical antidepressant activity.[1] The excretion of amitriptyline and nortriptyline into breast milk is still controversial in the literature, and the potential risk for the neonate remains unclear.[2-4]

The determination of the degree of transfer of drugs from the blood stream into the breast milk requires the availability of analytical methods for quantifying these drugs in both plasma and breast milk. The primary difference between breast milk samples and other body fluids usually analysed is that breast milk contains higher amounts of fatty acids and related lipids (3.8% on average).[5] The lipid materials can reduce extraction efficiency and cause analytical interference, especially when using gas liquid chromatography.[2] Breast milk also contains high levels of proteins (1% on average)[5] and tricyclic antidepressants are known to have high affinity to both plasma and milk proteins (amitriptyline is 95% bound to plasma proteins). Consequently, extraction of tricyclic antidepressants from milk requires an efficient method able to break the micelles of fatty acids and eliminate lipids.

Various methods, using either liquid phase or solid phase extraction and gas-liquid chromatography or HPLC, has been reported in the literature for determination of amitriptyline and its metabolites in plasma,[6-8] however, no methods describing the determination of these drugs in human milk are yet available. Pittard et al.[4] and Bader et al.[9] have applied to milk samples the commonly used gas liquid chromatography methods developed by Jørgensen[10] or Biggs et al.[11] for plasma samples, without any change. However, the quality parameters of the measurements from milk were not discussed. In 1980, Brixen-Rasmussen et al.[12] adapted the method from Jørgensen to milk samples, but multiple solvent extractions were necessary, reducing the extraction efficiencies and subsequently the limits of detection to 10 $ng.mL^{-1}$ and 30 $ng.mL^{-1}$ for amitriptyline and nortriptyline, respectively. Until now, no other attempt of extraction has been mentioned in the literature.

In this paper, a new method using a solid-phase extraction and a reversed phase liquid chromatography with a diode array detector (RP-HPLC-DAD), for the determination of amitriptyline and nortriptyline in human breast milk is reported.

## EXPERIMENTAL

### Chemicals

Amitriptyline·HCl, nortriptyline·HCl, and imipramine·HCl were obtained from Sigma-Aldrich Ltd. (Oakville, Ontario, Canada).

Acetonitrile, methanol, isoproylamine, and hexane were purchased from V.W.R. Scientific Ltee (Montreal, Quebec, Canada). HPLC water reagent was from Moquin scientific Inc. (Lachenaie, Quebec, Canada). Ammonium acetate and hydrochloric acid were from Sigma-Aldrich Ltd. (Oakville, Ontario, Canada).

Solvents and chemicals were all of analytical grade.



### Calibration and Reagent Solutions

A standard solution containing 1.5 $\mu g.mL^{-1}$ of amitriptyline and nortriptyline was prepared in methanol. The working internal standard (IS) solution contained 1 $\mu g.mL^{-1}$ of imipramide in methanol.

For the milk assays, a series of dilution was prepared in order to obtain the following concentrations of amitriptyline and nortriptyline: 60, 120, 180, and 300 $ng.mL^{-1}$.

Standard solutions were kept away from light and stored at 4°C for several months, without deterioration. Milk samples were stored at 4°C for a few days.

### Instrumentation and Chromatographic Conditions

HPLC-DAD analyses of amitriptyline and nortriptyline were performed on a HP-1100 system, from Hewlett-Packard (Kirkland, Quebec, Canada). The chromatographic system consisted of a vacuum degasser on line with a binary pump linked to a thermostatted column compartment via an autosampler. The detection was carried out using a diode array UV detector (200 – 400 nm). Drugs were separated on a Eclipse XDB-C18 reversed phase column (30 mm × 4.6 mm, 3.5 μm particle size) from Hewlett-Packard (Kirkland, Quebec, Canada).

The mobile phase was a mixture of acetonitrile and acetate ammonium 0.1M (33/67, v/v). A fresh mobile phase was prepared every 3 days. Before use,

the mobile phase was filtered through a 0.45 μm membrane from Gelma Sciences (Ann Arbor, Michigan, USA). The flow was set at 1.5 $mL.min^{-1}$. The column compartment was kept at a controlled temperature of 38°C ± 0.5°C. The pressure was monitored at 80 bars. Amitriptyline and nortriptyline were detected at 238 nm and imipramine was detected at 250 nm.

## Sample Preparation: Solid Phase Extraction (SPE)

One mL of HCl 0.02 M and 25 ng of imipramine (IS) were added to 250 μL of human breast milk. After vortex-mixing for 1 min, the mixture was sonicated in a ultrasonic bath for 45 min. The sample was then loaded onto a SPE column, Oasis C18 from Waters Corporation (Milford, Massachusetts, USA), which had previously been conditioned with 1 mL of methanol, 1 mL of 0.02 N HCl and 1 mL of water. The column was washed with 1 mL of water and dried under vacuum for 5 min. Then, it was washed with 2 × 1 mL of hexane and dried under vacuum for 5 min. One mL of methanol containing 1% of isopropylamine was used for the elution. The eluent was collected and dried in a Rapidvap™ evaporator (Labconco, Kansas City, Missouri, USA) at 45°C under vacuum, with 95% vortex speed. Finally, the sample was reconstituted into 1 mL of methanol and a 1 μL aliquot was injected into the HPLC system for analysis.

## Analytical Parameters

The within-day precision was calculated from repeated analysis (n=5) during one working day and the between-day precision was calculated from repeated analysis on 5 successive working days. The efficiency of the extraction procedure was evaluated by comparing peak area ratios to internal standard, obtained with and without extraction.

Calibration curves were constructed by plotting peak area ratios (compound/internal standard) versus standard concentrations, and the best relationship was determined by a least-squares linear regression analysis.

The accuracy was tested using a Student's T-test, comparing the mean concentration $C_m$ of a milk standard sample injected five times (n=5) calculated from the equation of the linear regression, with the theoretical target concentration $C_t$ of this sample. Accuracy was verified with a risk of 5% when $T=(C_m-C_t)/(SD/2) < T_{table}(\alpha=5\%,n-1)$, where SD is the standard deviation of $C_m$.

The limit of detection (LOD) was evaluated at a signal-to-noise ratio of 3.

## RESULTS AND DISCUSSION

Figure 1 shows a typical chromatographic profile of the analysis of amitriptyline and nortriptyline extracted from a human breast milk sample. Chromatographic conditions were optimised to obtain baseline separation of the two drugs and the internal standard. Retention times of amitriptyline, imipramine (IS), and nortriptyline were 1.32 min, 1.77 min, and 2.41 min, respectively. Resolution factors between peaks were calculated and found to be superior to 1.5.

SPE has been chosen for milk sample extraction and cleanup due to its simplicity and rapidity, as well as low solvent requirements, compared to liquid-liquid extraction. Furthermore, for practical and ethical reasons, it is often difficult to obtain large volumes of breast milk. SPE allows working on small volumes of sample. The extraction procedure described above required only 250 µL of milk. Before loading the sample onto the extraction cartridge, the milk was acidified with diluted HCl in order to ionise drugs, without degradation. The milk sample was sonicated in an ultrasonic bath in order to break the micelles of fatty acids and to break bonds between drugs and proteins. When the sample is loaded onto the SPE column, fatty acids and proteins are retained by the sorbent. Therefore, the column was washed with hexane to elute these contaminants. Then, the drugs were eluted by proton exchange with methanol containing 1% of isopropylamine.

The recoveries and the precision of the method are listed in Table 1. High mean recoveries were obtained for amitriptyline (92%) and nortriptyline (89%). Results indicate that extraction recoveries are independent of the concentrations



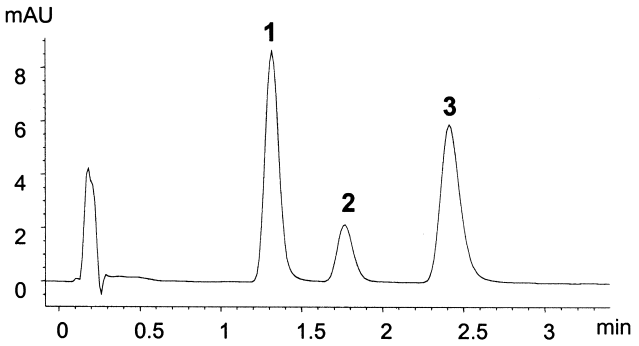


***Figure 1.*** Chromatogram of blank milk spiked with standard solution at 120 ng.mL$^{-1}$, registered at 238 nm. Peaks: 1=amitriptyline, 2=imipramine (IS), 3=nortriptyline.

***Table 1.*** Recoveries, Within- and Between-Day Precision (n=5)

| Concentration ($ng.ml^{-1}$) | Recovery (%) | Coefficient of Variation (%) | |
|---|---|---|---|
| | | Within-Day | Between-Day |
| *Amitriptyline* | | | |
| 60 | 91 ± 3 | 0.9 | 5.7 |
| 120 | 92 ± 6 | 1.1 | 2.5 |
| 180 | 90 ± 8 | 0.5 | 5.1 |
| 300 | 93 ± 9 | 0.3 | 6.5 |
| *Nortriptyline* | | | |
| 60 | 86 ± 10 | 1.3 | 5.8 |
| 120 | 91 ± 6 | 1.6 | 1.8 |
| 180 | 88 ± 9 | 0.4 | 3.7 |
| 300 | 92 ± 9 | 0.3 | 5.2 |



of amitriptyline (SD 7%) and nortriptyline (SD 8%). Within-day precision values were below 1.6%. Between-day coefficients of variation were higher than within-day coefficients, ranging from 1.8% to 6.5%. The modifications of milk composition during the storage under refrigeration at 4°C may contribute to these variations.[13] Nevertheless, the precision is sufficient enough for the determination of amitriptyline and nortriptyline in breast milk.

The calibration functions were $y = 0.0316\ x - 0.0737$ ($R^2$=0.9992) and $y = 0.0304\ x - 0.0773$ ($R^2$=0.9996) for amitriptyline and nortriptyline, respectively. Therefore, the response of the detector showed a good linearity for quantification of both drugs in the studied range of concentrations (60-300 $ng.mL^{-1}$). The limit of detection for amitriptyline and nortriptyline was of 1.9 $ng.mL^{-1}$. These values

***Table 2.*** Accuracy Using a T-Statistic*

| Compound | $C_t$=90 ($ng.ml^{-1}$) | | $C_t$=240 ($ng.ml^{-1}$) | |
|---|---|---|---|---|
| | $C_m$ | T | $C_m$ | T |
| Amitriptyline | 89 ± 2 | 1.3 | 243 ± 3 | 1.8 |
| Nortriptyline | 89 ± 1 | 2.3 | 242 ± 3 | 1.1 |

*$T=(C_m-C_t)/(SD/2)$; $C_m$ and SD: mean and standard deviation of concentrations measured from the equation of the linear regression (n=5); $C_t$: concentration of the standard sample. $T_{table}(0.95, 3)=2.8$.

are of the same order than those reported in the literature for plasma sample, analysed by liquid-liquid extraction and HPLC-DAD (5-10 $ng.mL^{-1}$,[14] 2 $ng.mL^{-1}$.[15]

Accuracy was tested with two concentrations within the calibration range. Table 2 displays the values of $C_m$ and T for two standard milk samples containing amitriptyline and nortriptyline at 90 $\mu g.mL^{-1}$ and 240 $\mu g.mL^{-1}$. The accuracy of the method is validated.

In conclusion, the solid-phase extraction procedure described here provides a simple and efficient preparation method for the determination of amitriptyline and nortriptyline in human breast milk. Since no method has been reported in the literature as being as simple, rapid, efficient, and sensitive as our SPE with HPLC-DAD procedure, the method described above could be useful for measuring the transfer of amitriptyline and nortriptyline in the breast milk and the potential risk of adverse effect or toxicity for the neonate.



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