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Simultaneous quantification of fluoxetine and norfluoxetine in colostrum and mature human milk using a 2-dimensional liquid chromatography–tandem mass spectrometry system

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ABSTRACT

A two-dimensional liquid chromatography system coupled to triple quadrupole tandem mass spectrometer (2D LC–MS/MS) was employed for the determination of fluoxetine (FLU) and norfluoxetine (N-FLU) in colostrum and mature milk by direct sample injection. With a run time of 12 min representing a gain in throughput analysis, the validated methods furnished selectivity, extraction efficiency, accuracy, and precision in accordance with the criteria preconized by the European Medicines Agency guidelines. With a linear range of 3.00–150 ng/mL for FLU and 4.00–200 ng/mL for N-FLU they were applied to the analysis of colostrum and mature milk samples from nursing mothers. The paper discusses the differences and similarity of sample preparation for this two sample matrices. The herein reported methods are an advance in sample preparation procedures providing waste reduction and a sustainable approach.

1. Introduction

Human breast milk is the ideal source of nutrition for infants and the American Academy of Pediatrics recommends exclusive breastfeeding for at least the first 6 months of life because it offers several benefits to both the infant and the mother [1,2].

The human milk usually classified as colostrum, transitional milk and mature milk contains high concentrations of proteins, lipids, and carbohydrates and the composition these components are drastically altered during the lactation period to reflect the infant's needs. Colostrum, the milk produced during the first few days of lactation, is significantly different from mature milk; in comparison, the protein level is significantly higher while the lactose content is lower; it has also high content of fat-soluble vitamins, minerals, and immunoglobulins. On the other hand, colostrum presents low concentration of lipids which increases gradually during the lactation [3].

Although breast milk is the most suitable food for the infant, the breastfed child may be exposed to drugs during maternal drug therapy and for this reason it is important to determine the extent of medication that can be transferred to the milk, and how this may affect the development of the infant [4,5].

Colostrum is a complete diet for the newborn and it plays an important role in protecting their immune system [6]. Thus, relating to drug transfer this neonatal period needs also to be addressed specially for drugs used for chronically diseases.

Incidentally, for many women, depressive disorders are common during pregnancy and in the early months after childbirth because of physiological hormonal changes [7]. Fluoxetine (FLU, Fig. 1A), a selective serotonin reuptake inhibitor (SSRI), is commonly prescribed for the treatment of depression in pregnancy and postpartum period [7]. It is extensively metabolized in the liver to its major active metabolite norfluoxetine (N-FLU, Fig. 1B), which is slightly more potent than its parent drug [8]. Although a number of LC methods have been reported for the simultaneous quantification of FLU and N-FLU in human plasma [9–15], only few of them have dealt with these analytes in human milk [16–18]. In regarding to sample clean up, as for other drugs, they usually follow adopted procedure used for other biofluids without considering that this is a more complex matrix [19]. Quantification of FLU in human milk in a multi-drug method has been recently reported [20] the sample preparation was, however, protein precipitation. Towards

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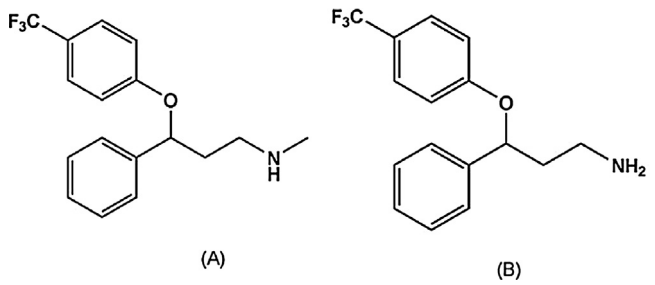

Fig. 1. Chemical structures of (A) Fluoxetine and (B) Norfluoxetine.

a sustainable approach for the sample preparation of this difficult biological matrix, we have reported the simultaneous enantioselective quantifications of FLU and N-FLU using a two-dimensional liquid chromatography system coupled to a triple quadrupole tandem mass spectrometer (2D LC–MS/MS) [21].

In the same line of producing conditions for direct sample injection and also for providing conditions of a routine evaluation of the transfer of FLU and N-FLU to breast milk, herein we report the quantification of FLU and N-FLU in colostrum and mature milk by the use of a 2D LC–MS/MS.

2. Materials and methods

2.1. Chemicals

LC-grade acetonitrile (ACN), methanol (MeOH) and 2-propanol were purchased from Mallinckrodt Baker (St. Louis, MO, USA). Water was purified using a Milli-Q system from Millipore (São Paulo, SP, Brazil). Fluoxetine hydrochloride (FLU) was donated by Vita Nova Institute (Hortolândia, SP, Brazil) and norfluoxetine hydrochloride (N-FLU) was obtained from Sigma-Aldrich (Steinheim, Germany), bovine serum albumin (fraction V powder minimum 98%) was purchased from Sigma (St. Louis, MO, USA), ammonium acetate (NH_4OAc) and other reagents were of analytical grade.

2.2. Instrumentation

The analyses were carried out using an Acquity UPLC® system coupled with a Xevo® TQ-MS (Waters, Milford, MA, USA). The chromatographic equipment consisted of a binary pump (BSM), a quaternary H-Class pump (QSM), an UV–vis detector, an autosampler model 2777C with one six-port Valco® valve and two six-port Everflow® valves for the automated column-switching. Mass spectrometric detection was carried out using an electrospray interface (ESI) operating in the positive ionization mode. MassLynx 4.1 software (Waters, Milford, MA, USA) was used for data acquisition and processing. The optimization of the ionization source and voltages on the lenses was done by IntelliStart tune mode.

2.3. Chromatographic parameters

The RAM-BSA C8 (30 × 2.1 mm I.D., Luna®, 10 μm, 100 Å) for size-exclusion of high molar mass matrix components was prepared as described previously [22]. The chromatographic analysis of analytes in mature milk and colostrum were performed using an Ascentis® Express phenyl-hexyl column (100 × 2.1 mm, 2.7 μm, Supelco, Bellefonte, PA, USA) and an Ascentis® Express C18 column (100 × 2.1 mm, 2.7 μm, Supelco, Bellefonte, PA, USA), respectively.

The column-switching system used for coupling of the RAM and the analytical columns was previously reported [21].

2.4. Mass spectrometry parameters

For the optimization of ionization conditions, full scan acquisitions were made to the specific mass ranges for individual compounds. For that, standard solutions of each compound (100 ng/mL) were continuously infused at a flow rate of 20 μL/min by a syringe pump into the mobile phase stream. The ESI–MS/MS was operated in the selected reaction monitoring (SRM) mode in which the protonated molecular ion was isolated and the fragment ions were monitored. Nitrogen was used as dessolvation gas at 800 L/h flow rate with dessolvation temperature set at 600 °C and the source temperature at 150 °C. The capillary voltage was set at 2.5 kV and the collision gas flow at 0.15 mL/min. For each analyte two SRM transitions were used, one for quantitation and other for confirmation. The cone voltage (CV) and collision energy (CE) were respectively: 310.1 > 44.0 (CV = 12 V; CE = 10 eV) and 310.1 > 148.1 (CV = 18 V; CE = 8 eV) for FLU; 296.1 > 29.9 (CV = 12 V; CE = 6 eV) and 296.1 > 134.1 (CV = 12 V; CE = 6 eV) for N-FLU.

2.5. Working solutions

A stock solution containing FLU (15.0 μg/mL) and N-FLU (20.0 μg/mL) was prepared in acetonitrile. From this stock solution were prepared, in water, at the following working solutions: 0.15, 0.75, 1.50, 2.25, 4.50, 6.75 and 7.50 μg/mL for FLU and 0.20, 1.00, 2.00, 3.00, 6.00, 9.00 and 10.0 μg/mL for N-FLU (to calibration) and 0.30, 3.00 and 6.00 μg/mL for FLU and 0.40, 4.00 and 8.00 μg/mL for N-FLU (to quality controls (QC) samples). Stock and working solutions were stored at −20 °C.

2.6. Mature milk sample preparation

The spiked mature milk samples were prepared as previously described [21]. In short: to each 445 μL of milk 10.0 μL of the appropriate working solution were added before vortex-mixing for 30 s. To allow to reaching steady-state equilibrium with the matrix components they were left to stand for 10 min. Then, 40.0 μL of 2-propanol and 5.00 μL of formic acid were added before centrifuging (13,420 *g*) for 15 min at 4 °C. From the middle layer formed, 200 μL were transferred to autosampler vials and 10.0 μL was injected onto the 2D LC–MS/MS system.

2.7. Colostrum sample preparation

Aliquots of 12.3 μL of the appropriate working solution were transferred to centrifuge tubes, followed by addition of 200 μL of ultrapure water and 250 μL of colostrum milk. After vortex-mixing (30 s), the samples were kept for 10 min at room temperature to allow a steady-state with the matrix components, treated with 150 μL of acetonitrile, 5.00 μL of formic acid and centrifuged at 13,420 *g* at 4 °C for 15 min. The middle aqueous layer was collected and 10.0 μL was subjected to LC–MS/MS analysis.

2.8. Method validation

The developed methods were validated in accordance to the European Medicines Agency guideline (EMA) [23] for both matrices.

2.8.1. Selectivity, carryover and matrix effect

Drug-free matrices samples, mature milk and colostrum samples spiked with FLU and N-FLU were prepared as described above and analysed to ensure the selectivity of the method.

Carryover was evaluated during each batch by injection of a blank sample after the analysis of the upper limit of the calibrators samples (150 ng/mL FLU and 200 ng/mL N-FLU) and after HQCs analyses (120 ng/mL FLU and 160 ng/mL N-FLU).