A Simple and Efficient HPLC Method for Benznidazole Dosage in Human Breast Milk

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Background: Due to migration, Chagas disease is a significant public health problem in Latin America, and in other nonendemic regions. The 2 drugs currently available for the treatment, nifurtimox and benznidazole (BNZ), are associated with a high risk of toxicity in therapeutic doses. Excretion of drug into human breast milk is a potential source of unwanted exposure and pharmacologic effects in the nursing infant. However, this phenomenon was not evaluated until now, and measurement techniques for both drugs in milk were not developed.

Methods: In this work, we described the development of a simple and fast method to quantify BNZ in human milk using a pretreatment that involves acid protein precipitation followed by tandem microfiltration, and reverse phase high-performance liquid chromatography/ultraviolet analysis. It is simple because it takes only 3 steps to obtain a clean extracted solution that is ready to inject into the high-performance liquid chromatography equipment. It is fast because a complete analysis of a sample takes only 36 minutes.

Results: Although the human breast milk composition is very variable, and lipids are one of the most difficult compounds to clean up on a milk sample, the procedure has proven to be robust and sensitive with a limit of detection of 0.3 μg/mL and quantization of 0.9 μg/mL. Despite a 70% recovery value, which could be considered a relatively low result, this recovery is reproducible (coefficient of variation <10%) and the analytical response under the linear range is very good (r^2 = 0.9969 adjusted). Real samples of human breast milk from patients in treatment with BNZ were dosed to support the validation process of the method.

Conclusions: The method described is fast, specific, accurate, precise, and sufficiently sensitive in the clinical context for the quantification of BNZ in human milk. For all these reasons, it is suitable for clinical risk evaluation studies.

Key Words: breast milk, benznidazole, HPLC, Chagas disease

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INTRODUCTION

Chagas disease, a protozoan infection caused by the kitenoplastid *Trypanosoma cruzi*, constitutes a major public health problem in Latin America. According to current data, there are >7 million infected people in the Americas[1] with an incidence of >40,000 new cases per year.[2]

Initial infections with *T. cruzi* take place mostly in children, by vector or congenital transmission.[3] As vector control improves, congenital transmission is rapidly becoming the main route of infection, highlighting the importance of the diagnosis of maternal infection. However, in view of the particular cultural and social context of endemic Chagas disease regions, this diagnosis is frequently associated with pregnancy screening or birth attendance.[4] Young women with Chagas disease who are diagnosed during pregnancy (a common occurrence) are not treated after they give birth because lactation is perceived as a contraindication to treatment of the mother (to avoid exposing the baby to unknown amounts of the drug through breast milk). Unfortunately, particularly in rural areas, difficult access to medical care and, in some cases, short interpregnancy periods may significantly decrease the chances that these women are actually treated. To further exacerbate the situation, the perceived contraindication to use antichagasic medication during lactation is not based on any pharmacologic data.

There are only 2 drugs currently available for the treatment of Chagas disease: benznidazole (BNZ) and nifurtimox (NFX). Both drugs are associated with high risks of toxicity, especially dermatologic reactions in adults,[5,6] but risks of toxicity are low in infants and children.[7] BZN is the most commonly used drug in South America for the treatment for Chagas disease, due to availability issues. (In Argentina, Public Health Programs mainly provide BNZ for Chagas treatment.)[8]

Human breast milk is a biological sample of great importance for the analysis of therapeutic drugs, as unwanted exposure through breast milk could result in pharmacologic effects in the nursing infant. The goal of breast milk drug

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analysis is to inquire the level of exposure of the neonate to a drug during lactation, and also to evaluate potential risks of maternal therapy. Evaluation of possible risks to neonates and infants requires careful consideration, and is not limited only to drug concentrations in the milk but should also include known or suspected pharmacokinetics and pharmacodynamics (eg, adverse drug reactions) of the drug in the newborn child, and the potential risks of discontinuation of breastfeeding, among other factors. For these reasons, the estimation of risks requires a knowledge of drug concentrations in breast milk to estimate infant exposure.

Drug transfer from plasma into breast milk occurs primarily by passive diffusion depending on molecular weight, drug ionization, lipid solubility, protein binding, and mammary blood flow.[9–12] However, it remains unknown to what extent NFX or BNZ are transported into breast milk. Without this knowledge, most physicians who treat adult patients with Chagas disease refuse to treat women during lactation. In fact, in none of the few formularies that include BNZ (eg, World Health Organization model formulary; http://apps.who.int/medicinedocs/documents/s16879e/s16879e.pdf) indicated that it is deemed compatible with breastfeeding. At best, the formulary provides no information on breastfeeding or states that it is not compatible due to lack of sufficient data.

However, based on a theoretical analysis, transference of NFX into breast milk is expected to be limited and unlikely to lead to a significant exposure of the breastfed infant.[4] To date, there are no theoretical or experimental data about human breast milk BNZ transference. Accordingly, safety and scientific reasons, and regulatory requirements demand the evaluation of the potential exposure of children to antichagasic drugs through breast milk.

The lack of information on BNZ transfer into breast milk, and the potential public health impact that the treatment of lactating women with Chagas disease could provide, led us to plan a clinical trial to study the degree of transfer of BNZ into breast milk. In this context, a suitable procedure for sample pretreatment with a subsequent analytical method needs to be developed for reliable drug determination in this biological matrix characterized by its complexity and variability.

Many alternative extraction or clean-up methods have been proposed for the analysis of drugs or organic contaminant in milk including immunoaffinity methods,[13] liquid–liquid extraction,[14–17] liquid-phase microextraction,[18] C_{18} solid phase extraction (SPE),[19–22] ion-exchange/reversed-phase SPE,[23] and QuEChERS methodology.[24] Other direct pretreatment methods have also been reported, such as simple acidic/organic solvent precipitation[25–27] and ultrafiltration.[28,29] Exceptionally, the use of an internal surface reversed-phase column has also been described, originally created for the specific and direct analysis of drugs in serum without extensive sample preparation, to effect a true direct injection of milk into a reversed-phase system.[30] However, no analytical methods have been lately reported for the BNZ analysis in human breast milk, and only a few methods were tested in other human matrices.[31,32] The aim of this work is to develop an analytical method to quantify BNZ in human breast milk for further use in clinical research, and for routine clinical use in therapeutic drug monitoring.

EXPERIMENTAL

Materials, Reagents, and Equipment

BNZ was obtained from Hoffmann-La Roche Ltd. (Buenos Aires, Argentina). Trichloroacetic acid proanalysis was purchased from Biopack (Argentina). Dimethyl sulfoxide proanalysis was obtained from Anedra (Argentina). Chromatographic grade demineralized water (<0.2 μS) was obtained in our laboratory with ionic interchange resins. Acetonitrile high-performance liquid chromatography (HPLC) grade was used from JT Baker (Mexico).

For filtering procedures, 3-mL polypropylene syringes, 2 polypropylene filter holder in tandem, consecutively with 13-mm nylon hydrophilic membranes of 0.80- and 0.45-μm diameter pore (Osmonics Inc) were used.

A Rolco 2036 centrifuge and a certificate analytical balance 0.1 mg (OHAUS-Pioneer) were used. All micropipettes were calibrated before use. All HPLC solvents were degassed with a vacuum pump (Pascal-Czerweny). HPLC was carried out in an HPLC Merck-Hitachi LC-6200A chromatograph connected to a Merck-Hitachi ultraviolet/visible L-4250 detector. Separation was carried out at room temperature using a Merck Lichrospher-100 C_{18} column (5 μm, 100 × 4.6 mm). The samples were injected manually (20-μL sample loop). Peaks areas were integrated automatically by a Merck-Hitachi D-2500 Chromato-Integrator.

Stock and Working Solutions

Stock control solution was prepared with 91.7 mg of BNZ solid drug in 5000 μL of dimethyl sulfoxide to complete dissolution and then accurately diluted to 25.00 mL with acetonitrile to obtain a 3668 μg/mL stock solution that was stored at 4°C.

Three different dilutions (1/10, 1/50, 1/100) were made from the stock solution in aqueous mobile phase to obtain 10.00 mL of 3 standard working solutions (SWS) of 366.80, 73.36, and 36.68 μg/mL, respectively. The standard solution curve was made at 6 duplicated levels of 0.50, 1.00, 2.50, 5.00, 10.00, and 15.00 μg/mL by preparing a suitable dilution from SWS.

Breast Milk Samples and Pretreatment Procedure

Preparation of Standard Spiked Breast Milk Samples

Drug-free breast milk samples were obtained from healthy volunteers without concomitant medication or known drug abuse and stored in polypropylene flasks with lid at −20°C until analysis. In the same way as explained above, the SWS were also used to obtain the spiked breast milk curve for triplicate 1000-μL samples. Concentrations of 0.50, 1.00, 2.50, 5.00, 10.00, and 15.00 μg/mL were prepared from a dilution of a volume of SWS into drug-free breast milk.

Patients' Breast Milk Samples

Seventeen breast milk samples of 10 different patients were obtained from lactating mothers with Chagas disease treated with BNZ (5–10 $mg \cdot kg^{-1} \cdot d^{-1}$), who were enrolled in

a clinical study approved by both ethical and research review committees of Ciudad Autónoma de Buenos Aires Children's Hospital Ricardo Gutierrez, Buenos Aires city, Argentina.[33] All of them were not taking abuse drugs or any other medication except for BZN. Around 5 mL of breast milk per patient was collected in polypropylene flasks with lid, without additives.

Pretreatment of Breast Milk Samples

The 1000-μL spiked breast milk samples and milk samples from patients were deproteinized by adding 1/10 sample volume (100 μL) of an aqueous solution of trichloroacetic acid (30% vol/vol). After vortexing for 1 minute and sonicating for 10 minutes, the samples were filtered and centrifuged at 8000 rpm for 20 minutes to obtain an ultrafiltrate of breast milk.

Chromatographic Analysis

The obtained ultrafiltrated samples were directly injected into the HPLC column. HPLC analysis was performed by isocratic elution with a flow rate of 1.2 mL/min. The mobile phase composition was water/acetonitrile (80:20 vol/vol). All solvents were filtered through a 0.45-μm nylon membrane before use and degassed with a vacuum pump.

The wavelength was set at the maximum absorption value of 313 nm. A value of 0.030 absorbance units (au) threshold was used. For the calibration curve, 6 levels at 0.50, 1.0, 2.5, 5.0, 10.0, and 15.00 μg/mL were used for the spiked breast milk each one by triplicate. In the same sense, as a blank control, 3 of the 7 free-drug breast milk samples used for the ruggedness study were analyzed. All concentrations are related to the expected real samples dosage. The peak area was plotted against the known concentration to obtain the calibration curve and the regression parameters. Quantitative analysis was performed by the external standard procedure.

Validation Procedures

Limit of detection (LOD) was determined according to the International Union of Pure and Applied Chemistry definition of 3.3 S_b/b, where S_b is the intercept standard error and b is the slope. The lower limit of quantization (LOQ) was determined as $9S_b/b$. Selectivity was tested with analyses of blank breast milk samples obtained from 7 patients before the BNZ treatment, without concomitant medication known. The highest spiked breast milk level was defined based on the expected concentrations in probe samples. Linear ranges were assumed between lower LOQ and highest spiked breast milk sample on calibration curve. Accuracy and precision were determined from the triplicates of the calibration curve on the 5 levels that were inside the linear range. The total uncertainty was calculated as the sum of accuracy and precision. Sensitivity calculation was estimated as the product of total uncertainty with LOQ. Recovery and matrix effects were evaluated for comparison between standard solution curve and spiked breast milk curve data inside the linear range of the second one. Long-term stability was investigated with 3 random SWS stored for 3 months at 4°C and with 2 spiked breast milk samples at 5.0 and 15.0 μg/mL stored for 1 month at −20°C.

Ruggedness Study

For all the reasons mentioned above, it is necessary to investigate if there is a relationship between lipoprotein (LP) and lipidic (L) content in milk and the BZN recovery as a ruggedness measure. The LP and L contents of 7 free-drug milk samples were determined. LP content measurements were carried out in microhematocrite capillary glass tubes filled with breast milk. The capillary tubes were centrifuged at 8000 rpm for 5 minutes to obtain 2 phases: lipoproteic and aqueous. L content measurements were carried out in the same conditions with a previous addition of 100 μL of sulfuric acid (98% wt/wt) to 1000 μL breast milk in a 1.5-mL eppendorf tube. After shaking for 1 minute, microhematocrite capillary glass tubes were filled. The capillary tubes were centrifuged at 8000 rpm for 5 minutes to obtain 3 phases: lipoproteic, aqueous, and a protein precipitate. LP and L percentages of the total liquid column length were determined with a 0.05-mm sensitivity lineal caliber and a correction of volume for the L content.

On the other hand, a volume of BNZ SWS was added to 1000 μL each of the same 7 drug-free breast milk samples to obtain a final 5.00 μg/mL BNZ concentration. Spiked breast milk samples were pretreated and analyzed by HPLC as mentioned above. Recovery percentage was calculated relating to the standard calibration curve.

RESULTS

Preanalysis handling time was estimated in 30 minutes. In the instrumental procedure, the retention time for BNZ was 4.32 ± 0.18 minutes. Total HPLC run time was 8.00 ± 0.18 minutes per sample. Typical chromatograms for BNZ in SWS (A) and breast milk (B and C) are shown in Figure 1. BNZ in SWS showed no decomposition products in the chromatogram and no significant loss during the analytical procedure and after 3 months of storage at 4°C. In the same sense, BNZ in spiked breast milk samples showed no significant loss of response in the chromatogram after 1 month of storage at −20°C. On the other hand, in the breast milk blank controls analyzed, there was no chromatographic signal that could be an interference for BZN peak.

The correlation curve obtained for spiked samples was ($y = 2441x = 44$), where x is the BNZ concentration and y is the instrumental response. Good linearity was obtained for BNZ with $r^2 = 0.9969$ (adjusted). Calculated LOD and LOQ were 0.3 and 0.9 μg/mL, respectively. This implies a linear range between 0.9 and 15 μg/mL. Recovery at the 5.0 μg/mL level was 70%.

Accuracy and precision were 6.6% and 2.1%, respectively. The total uncertainty was <10%. The sensitivity was 0.1 μg/mL, below the LOD value. The residual-lag plot suggests that the variance is not random and that the total error is the same in the entire concentration range.

The LP determinations showed an average of 4.0% with an SD of 2.3% in the range of 1.9%–8.7%. The L determinations showed an average of 3.5% with an SD of 1.8% in the range of 1.8%–6.9%. Analysis of variance over correlation between LP and L dosages showed an F value of 40.165, implying a linear correlation with a 99.95% of significance.

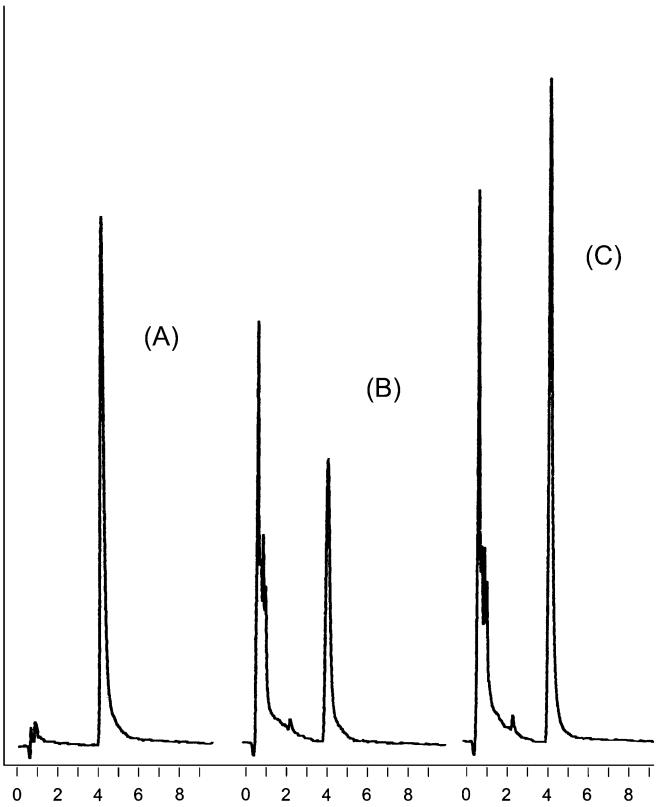

FIGURE 1. Typical chromatogram for (A) standard solution at 5.0 μg/μL, (B) free-drug spiked sample at 5.0 μg/μL, and (C) real sample measure at 9.2 μg/μL.

The mean percentage recovery was 70% with an SD of 11%. Analysis of variance for a correlation between LP and L with the percentage recovery showed *F* values of 0.457 and 0.470, respectively. This implies the absence of correlation between LP and L with percentage recovery with 99.99% significance.

Dosage on the 17 clinical samples showed good performance of the technique, with levels between undetectable and 9.8 μg/mL. These measures show a mean of 4.1, a coefficient of variation of 0.58, and a variance of 5.66. The first quartile (Q1) and third quartile (Q3) are 3.1 and 5.0, respectively.

DISCUSSION

Milk is a complex analytical matrix. The proteic fraction of milk contains numerous hydrophilic proteins that are difficult to separate by precipitation. Also, lipid globules can retain lipophilic molecules, leading to lower recoveries in the extraction procedure. These facts may significantly complicate analysis. On the other hand, if lipids are not removed from the sample, they can also deteriorate the chromatographic column used for the analysis.[14]

Human breast milk may vary significantly in composition and concentration in an individual and between individuals during a short period of time because of multiple reasons,[18,34,35] which determine large interindividual and intra-individual variations in samples.

The fatty-rich and lipoproteic phases can be removed through tandem filtration, but drugs may be strongly bound to these phases. This binding may be partially disrupted by sonication and filtration. Despite a 70% recovery value, which could be considered relatively low, this recovery is reproducible and the analytical response under the linear range is excellent for clinical applications. Moreover, we did not observe statistical correlations between lipid or LP content and recovery, suggesting a rugged methodology.

To find a suitable methodology to measure BZN in breast milk, many different pretreatment techniques, as liquid–liquid extraction, SPE, and QuEChER, were tested. All of them showed lower recovery values comparing with the tandem filtration technique and most of them needed bigger volumes of sample for the pretreatment. Moreover, all of them required multiple steps. In our validated method, the single precipitation step followed by a tandem ultrafiltration procedure was adequate and very simple compared with the literature reports. In this procedure, the first filter (0.80 μm) retains higher particles and prevented the second membrane (0.45 μm) from getting saturated. To our knowledge, a simple tandem ultrafiltration procedure was not previously reported for milk or other biological matrices. Moreover, this method shows ecocompatibility because it is carried without organic solvents.

On the mobile phase selection, different proportions of methanol, acetonitrile, and water were tested. The mobile phase to achieve optimum retention and sensitivity was found to be water/acetonitrile. Flow rates between 0.8 and 1.5 mL/min were tested, and 1.2 mL/min was found to be the rate with an optimal *S/N* ratio and a reasonable separation time.

In a limited but not few numbers of measured real samples, founded values were mainly into the linear range. Only two samples had no detected drug. However, these samples have no clinical significance to evaluate the safety of medication during lactation, because the risks could be significant only at values above that of our LOD method. Moreover, these measures were consistent with the expected dosage, assuming similarity with reported therapeutic plasma concentrations. However, these results are preliminary information that is not really able to assess the transfer of the drug. But in the same way, these data clearly show clinical validity of the method.

CONCLUSIONS

We report a reverse phase HPLC analysis of BNZ in human breast milk, with a simple pretreatment step involving acid protein precipitation followed by tandem microfiltration. The method described is fast, specific, accurate, precise, and sufficiently sensitive in the clinical context for quantification of BNZ in human milk.

The described method has also been validated. The method has proven to be robust and sensitive with LOQ of 0.9 μg/mL. The standard curve of the described method adequately covers the range of expected concentrations. To date, the method has been shown to be very rugged as milk

samples have been analyzed using a single HPLC analytical column without any apparent loss of sensitivity or resolution.

Through dosage in some but significant real clinical samples, we can infer that the method is adequate for clinical use and to evaluate drug transfer through breast milk to the nursing infant.

In the next step, we will address the issue of infant exposure risk. This must not only develop systematic measures in breast milk but dosage plasma in mothers and infant dose estimation are also required, into a protocol of whole clinical study. In this sense, the described technique is currently in use for the measurement of BNZ in clinical samples of lactating women treated for Chagas disease in a clinical trial (registry: clinicaltrials.gov # NCT01547533).

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