

## Determination of Benznidazole in Human Dried Blood Spots by Liquid Chromatography–Mass Spectrometry to Monitor Adherence to *Trypanosoma cruzi* Infection Treatment in Infants and Children

Jeremiah D. Momper,<sup>1\*</sup> Nathan J. Hanan,<sup>1</sup> Steven S. Rossi,<sup>1</sup> Mark H. Mirochnick,<sup>2</sup> Maria Luisa Cafferata,<sup>3</sup> Antonia Lavenia,<sup>4</sup> Isolina Flores,<sup>4</sup> Luz Gibbons,<sup>5</sup> Alvaro Ciganda,<sup>3</sup> Sergio Sosa Estani,<sup>5,6</sup> Edmund V. Capparelli,<sup>1</sup> and Pierre Buekens<sup>7</sup>

<sup>1</sup>University of California, San Diego, La Jolla, California; <sup>2</sup>Boston University School of Medicine, Boston, Massachusetts; <sup>3</sup>Unidad de Investigación Clínica Montevideo (UNICEM), Montevideo, Uruguay; <sup>4</sup>Ministerio de Salud Pública de la Provincia de Tucumán, Tucumán, Argentina; <sup>5</sup>Instituto de Efectividad Clínica y Sanitaria (IECS), Buenos Aires, Argentina; <sup>6</sup>Instituto Nacional de Parasitología “Dr. Mario Fatała Chaben,” Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Administración Nacional de Laboratorios e Institutos de Salud “Dr. Carlos G. Malbrán” (ANLIS), Buenos Aires, Argentina; <sup>7</sup>School of Public Health and Tropical Medicine, Tulane University, New Orleans, Louisiana

**Abstract.** Medication adherence is critical to the effectiveness of benznidazole (BZ) therapy for the treatment of Chagas disease. Assessing BZ adherence using traditional plasma sampling methods presents numerous challenges in resource-limited settings. Dried blood spot (DBS) sampling of BZ can be used to overcome logistical barriers and provides a less invasive method for assessing BZ levels. A BZ DBS assay using liquid chromatography–tandem mass spectrometry was developed and applied to a clinical study of infants and children being treated with BZ for *Trypanosoma cruzi* infection in Argentina. The assay was validated over a concentration range of 9.8–5,000 ng/mL. Inter-assay and intra-assay measures ranged from –2.9% to 2.7% and 0.5% to 8.3% for accuracy and from 3.5% to 12% and 1.6% to 13.6% for precision, respectively. The mean recovery of BZ was greater than 91%. Partitioning ratios for DBSs/plasma ranged from 0.95 to 1.02. A cohort of 10 infants and six children with *T. cruzi* infection being treated with BZ had median BZ concentrations of 1.2 (IQR 0.29, 2.14) µg/mL with seven of 65 (11%) samples above the BZ treatment goal of 3 µg/mL for adults. The reported DBS assay is a simple and accurate method for the quantitative measurement of BZ that can be applied to facilitate urgently needed clinical studies of BZ for the treatment of Chagas disease and assess BZ adherence in resource-limited settings.

### INTRODUCTION

Chagas disease is a parasitic infection caused by *Trypanosoma cruzi* that affects an estimated six million people worldwide, with most of the infections occurring in Latin America.<sup>1</sup> However, it has been increasingly detected in non-endemic countries, including the United States, Canada, Europe, Japan, and Australia.<sup>1–5</sup> In addition to vector-borne transmission, *T. cruzi* may also be passed from mother to child during pregnancy or childbirth, by blood transfusions and organ transplants from infected donors, or by contaminated food.

The pharmacotherapy of Chagas disease has changed very little since the development of the trypanocidal agents nifurtimox and benznidazole (BZ).<sup>6</sup> Benznidazole has largely been the drug of choice and has been shown to be effective in the acute and early chronic phases of the disease.<sup>7–9</sup> However, a high incidence of adverse events and associated non-adherence has contributed to limiting its usefulness.<sup>10</sup> The lack of efficacy of BZ in patients with chronic infection and in patients with cardiomyopathy secondary to Chagas disease was recently demonstrated in the long-awaited release of the BENznidazole Evaluation for Interrupting Trypanosomiasis (BENEFIT) trial, a randomized, placebo-controlled study designed to evaluate the safety and efficacy of BZ.<sup>11</sup> Although the results of the BENEFIT study showed that BZ did not significantly reduce cardiac clinical deterioration over 5 years of follow-up in patients with cardiac disease established

before treatment, the reductions in parasite detection in serum samples has helped refocus researchers to identify and treat young people who are still in the early stages of illness.<sup>12</sup> The results of the BENEFIT trial demonstrate the critical need for studies evaluating the efficacy of BZ therapy in all populations susceptible to Chagas disease. An improved understanding of BZ pharmacology in target populations, especially pediatric patients and women of reproductive age, is critical to the success of future trials.

Two recent population pharmacokinetic studies in pediatrics<sup>13</sup> and adults,<sup>14</sup> as well as a single- and multiple-dose study in healthy volunteers,<sup>15</sup> and a meta-analysis of single-dose studies,<sup>16</sup> have provided important information on the disposition of BZ. However, more research is needed to define optimal dosing regimens in both adult and pediatric populations, and to elucidate differences in BZ exposure–response relationships between these two populations. Thus, there is a need to develop a robust, economical, and logistically feasible assay that can determine systemic BZ concentrations and monitor adherence in susceptible populations in endemic areas where resources are limited.

Whole blood specimens for pharmacokinetic analysis are typically sampled by venipuncture. The blood is then further processed by centrifugation to isolate serum, plasma, red blood cells, or peripheral blood mononuclear cells. However, these methods present logistical challenges (e.g., immediate processing, refrigeration, freezing, and temperature-controlled packaging). Preservation of these matrices in the time period between sampling and eventual analysis can be onerous and impractical in resource-limited environments with endemic distribution of this disease. The use of dried blood spots (DBSs), which require as little as 5 µL of whole blood when measured with a liquid chromatography–tandem

\* Address correspondence to Jeremiah D. Momper, Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Dr., MC 0657, La Jolla, CA 92093-0657. E-mail: jmomper@ucsd.edu

mass spectrometry system,<sup>17</sup> has the potential to provide a practical methodology to measure BZ exposure in resource-limited areas. In addition to small sample volumes, the DBS assay offers numerous advantages over the assay of conventional serum specimens, including less invasive collection (finger or heel prick in lieu of venipuncture), easier preparation (no centrifugation or refrigeration), and simpler storage and shipment to bioanalytical laboratories (no need for freezers or dry ice), and antimicrobial properties of the DBS sample allow for the avoidance of biohazard shipping procedures.<sup>18,19</sup> Preformulation studies of BZ have shown that it is thermally stable up to 234°C,<sup>20</sup> which bodes well for DBS sample collection, storage, and transport of specimens from endemic regions to analytical laboratories that are not within close proximity, especially during summer months.

We describe the development and validation of a liquid chromatography-tandem mass spectrometry-based method for the quantification of BZ in human DBSs, which may be an appropriate matrix for assessing BZ pharmacokinetics, exposure-response relationships, and adherence in resource-limited clinical settings. The method was applied to DBS samples collected in a small clinical study of infants and children receiving BZ treatment for *T. cruzi* infection.

## MATERIALS AND METHODS

**Clinical study design.** An observational prospective study was conducted in Tucuman Province, Argentina, from November 2012 to July 2014 as a supplemental project of a study on congenital transmission of *T. cruzi*.<sup>21,22</sup> The population consisted of infants and their siblings who were infected with *T. cruzi* and receiving BZ from their clinical care provider. The study coordinator educated mothers on how to accurately dose BZ and observed the infant's first dose to ensure accuracy. Benznidazole tablets were split as necessary to provide an approximate daily oral BZ dose of 5 mg/kg divided twice daily (i.e., 2.5 mg/kg given every 12 hours). If there was a change in the infant's weight between visits, the dose was adjusted. Dried blood spot samples were collected by fingerprick at random times relative to dose administration during biweekly home visits for the duration of *T. cruzi* therapy to assess BZ levels. Following the fingerprick, blood was allowed to flow naturally without manipulation and drops of free-flowing blood were gently touched to filter paper to fill at least two of four preprinted circles (approximately 100  $\mu$ L of whole blood each). The dried blood spot samples were stored at -20°C in sealable plastic bags with desiccant and then shipped at room temperature to the University of California, San Diego Pediatric Pharmacology Laboratory. On receipt, the samples were again stored at -20°C until analysis.

Clinical and sociodemographic characteristics of participants and additional data, including date and approximate time of administration of the last three doses and food intake around the time of dosing, were collected on case report forms. Data were collected prospectively and in local language. All participating women (for infants and children aged less than 7 years) and siblings provided informed consent or assent to participate in the study. The study was approved by the Tulane University Institutional Review Board and the Ethics Committees of the Centro de Educación Médica e Investigaciones Clínicas "Norberto Quirno," Argentina.

**Method development and validation.** Blood collection cards (Whatman Protein Saver 903) were purchased from Whatman, Inc. (Piscataway, NJ). The benznidazole reference powder was purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography-grade water with 0.1% formic acid, methanol (MeOH), and methyl tert-butyl ether (MTBE) were purchased from Thermo-Fisher Scientific (Waltham, MA). All other chemicals and solvents were of highest purity available from commercial sources and were used without further purification.

**Preparation of calibrators and controls.** Dried blood spots for calibration, precision, accuracy, recovery, and stability were prepared from methanolic stock BZ standards. Benznidazole (1 mg/mL) was diluted 1:200 in a total volume of 10 mL of heparinized whole blood to give a concentration of 5,000 ng/mL. The other calibration curve standards were made through serial 1:2 dilutions with heparinized whole blood to create calibration samples of 5,000, 2,500, 1,250, 625, 312.5, 156.3, 78.1, 39.1, 19.5, and 9.8 ng/mL. Controls were prepared using a similar method with BZ standards, prepared from powders from a different lot number, at concentrations of 4,500, 900, 180, 36, 12.5, and 9.8 ng/mL in heparinized whole blood; 100  $\mu$ L of the calibration standards or controls was spotted onto blood collection cards, dried overnight at room temperature, and then stored at -20°C in sealable plastic bags with desiccant and a humidity indicator card.

**Preparation of assay samples.** The frozen blood collection cards, containing dried whole blood spots representing approximately 100  $\mu$ L of whole blood, were thawed at room temperature. Two-quarter-inch discs were punched within the preprinted circumference, one at the 8 o'clock position and the other at the 2 o'clock position, with each punch made from a card area that represented 100% coverage with dried blood. Each DBS was visually inspected for layering (double-spotting) and no punches were made in these areas. Disc punches were then placed in a capped microcentrifuge tube with 1 mL of elution buffer (50% MeOH:50% MTBE). The microcentrifuge tubes were vortexed for 30 seconds and allowed to elute for 2 hours at room temperature with gentle agitation using a rotary mixer at 100 revolutions per minute (RPM). Following centrifugation for 10 minutes at 10,000 RPM, the supernatant was transferred to a new microcentrifuge tube and reduced to null volume under a stream of purified N<sub>2</sub> at 37°C. This residue was reconstituted with 60  $\mu$ L (70 MeOH:30 0.1% formic acid in water), vortexed for 30 seconds, and then centrifuged at 13,000 RPM for 10 minutes, before transfer to a 400- $\mu$ L conical glass insert within a 1.5-mL glass autosampler injection vial.

**Liquid chromatography-tandem mass spectrometry methodology.** The liquid chromatography-tandem mass spectrometry system consisted of an AB Sciex API4000 MS/MS (Framingham, MA), using a positive-mode electrospray ionization source, in conjunction with an Agilent 1100 Series LC module. Analyst 1.6 (Build 3773) software controlled instrumentation and generation of quantitative results. The analytical column was a reverse-phase C-18 column (MAC-MOD Ace 5 C-18, 15 cm  $\times$  2.1 mm) with a compatible pre-column filter (MAC-MOD #MMC-210). Precursor/product transitions (*m/z*) in the positive ion multiple reaction monitoring mode were 261/255 for BZ quantitation and 261/258 for qualification. Source parameters were CAD 12, CUR 20, GS1 40, GS2 60, IS 5500, TEM 300, and EP 10 for both transitions.

The injection volume was 20  $\mu$ L, and the LC flow was 300  $\mu$ L/minutes isocratic at 40% 0.1% formic acid in water: 60% MeOH, which resulted in retention times of 2.5 minutes for BZ. Quantitation of BZ was performed by using external calibration standards to generate a curve using a least-squares linear regression algorithm to plot the peak area versus concentration with 1/response weighting. Linearity was verified using estimates of the correlation coefficient ( $r$ ), wherein  $r$  was required to be  $\geq 0.99$ , for calibration curve acceptance. Additional calibration curve acceptance criteria were that the mean back-calculated values for the 10 standards had to be within 15% of the nominal values, except for the lowest standard (9.8 ng/mL), which had to be within 20% of the nominal value.

**Limits of quantitation.** The limits of quantitation are the lowest and highest points on the calibration curve that could be accurately and reproducibly quantified. For this validation, the lowest calibration curve concentration was 9.8 ng/mL and the highest was 5,000 ng/mL.

**Precision and accuracy.** The precision and accuracy of this method was validated by analysis of the human DBS control samples prepared at the lower limit of quantitation (LLOQ) and at five additional concentrations spanning the calibration range. Precision was defined as the percent coefficient of variation (CV%) of each control sample after a series of replications using the equation:

$$CV\% = (SD/Mean) \times 100\%.$$

Accuracy was defined as the percent deviation (%DEV) from the theoretical value of each control sample using the following equation:

$$\%DEV = \left( \frac{[\text{Mean observed concentration} - \text{theoretical concentration}]}{\text{Theoretical concentration}} \right) \times 100\%.$$

The acceptance criteria for validation of the method require the means of the control samples to have a CV% and %DEV of  $\leq \pm 15\%$ , except for the LLOQ, which must be  $\leq \pm 20\%$ .

**Intra- and inter-assay precision and accuracy.** To assess the within- and between-assay precision and accuracy, three aliquots of each control sample were evaluated on each assay day for 3 days, during a 2-week time period.

**Stability.** Several aspects of BZ stability were examined. Stock solutions of BZ in MeOH (1 mg/mL) were evaluated after storage at  $-20^\circ\text{C}$  for 4 and 20 months. The injection buffer matrix stability was determined by reinjection of two control sample concentrations (4,500 and 36 ng/mL) after storage in autosampler vials at room temperature for 38 and 72 hours. The long-term storage stability of BZ DBS samples was determined at  $-20^\circ\text{C}$  by analysis of three replicates of two control sample concentrations (180 and 36 ng/mL) after storage for 3, 19, and 21 months.

**Recovery.** Recovery was determined in triplicate at two concentrations (900 and 39 ng/mL) by comparing the mean area observed in eluted DBS BZ peaks with that found in the unspotted sample (as measured in the injection buffer).

**Specificity and selectivity.** The susceptibility of this assay to interference by biogenic constituents in blank DBSs was evaluated by measuring the MS/MS response to the DBSs made from six different lots of drug-free heparinized

whole blood. Another experiment evaluated whether any of a mixture of 49 potentially concomitant drugs (all at 10,000 ng/mL), which were included in the preparation of DBS controls containing 900 ng/mL BZ, were able to produce perturbations in the MS/MS response. The consistency of the ratio between the two most abundant product ions ( $m/z = 255$ , the "quantitation" ion and  $m/z = 258$ , or the "qualification" ion) was evaluated to define the acceptance criteria for peak authenticity. The accuracy of liquid chromatography-tandem mass spectrometry peak identification is strengthened by use of a "qualifier" (QI) product ion, assuming its formation is consistent across the dynamic range of the assay. We, therefore, measured the intra-day precision (consistency) of the ratio between QI and Qn ( $m/z = 258/m/z = 255$ ) for six control concentrations.

**Partitioning ratios.** To evaluate the accuracy of DBSs as a surrogate matrix for plasma in the determination of BZ, the relative measured response of the BZ quantitation ion (Qn) was compared between plasma, dried plasma spots (DPSs), and DBSs. Fresh whole blood was prepared at each of the six quality control (QC) concentrations and allowed to incubate at  $37^\circ\text{C}$  for 1 hour with gentle rocking. Aliquots of each were taken to prepare plasma, DPS, and DBS samples. The samples were frozen at  $-20^\circ\text{C}$  for 7 days before extraction and MS/MS analysis. In only these partition ratio experiments, entire blood or plasma spots were extracted.

**Pharmacokinetic simulations.** Monte Carlo simulations were performed using a previously reported population pharmacokinetic model to predict BZ DBS levels in our patient population.<sup>23</sup> Concentration profiles for 540 virtual infants and children from 1 day of life to the age of 16 years were simulated. Each virtual infant was assigned 16 BZ sampling time points ranging from 0.25 to 24 hours post-dose (8,100 total observations). Sampling time points were extended past the 12-hour dosing interval to include BZ levels for patients who did not receive BZ doses on time. Age and weight distributions and BZ dosing were distributed to match the characteristics of our clinical study population. The target therapeutic trough level was defined as a minimum of 3  $\mu\text{g/mL}$ .

## RESULTS

**Clinical population.** Ten infants and six children with *T. cruzi* infection receiving BZ treatment were enrolled and had BZ DBS levels evaluated (Table 1). Three infants had positive parasitological examinations at birth or at 4–8 weeks after birth, seven infants had a positive rapid test and an ELISA test at 10 months, and the six children were siblings of study infants with a positive rapid test and an ELISA test during household screening. The 16 cases enrolled in the study finished treatment; one infant abandoned treatment after the third dose but was retreated and completed the treatment. The ages of participants ranged from 15 days to 15 years with a median age (IQR) of 12.9 (11.1, 47.4) months.

**Method validation and performance.** *Intra- and inter-day accuracy and precision.* The assay was validated over a concentration range of 9.8–5,000 ng/mL. Intra- and inter-assay precision and accuracy results are shown in Table 2. Inter-assay and intra-assay measures ranged from  $-2.7\%$  to  $2.7\%$  and  $0.8\%$  to  $8.3\%$  for accuracy and from  $3.5\%$  to  $11.9\%$  and  $1.1\%$  to  $13.5\%$  for precision, respectively.

TABLE 1  
 Patient characteristics

	Index subject (N = 10)	Siblings (N = 6)	Total (N = 16)
	n/N (%)	n/N (%)	n/N (%)
Age at enrollment			
< 1 month	2/10 (20.0%)	0/6 (0.0%)	2/16 (12.5%)
1–11 months	3/10 (30.0%)	0/6 (0.0%)	3/16 (18.8%)
1–4 years	5/10 (50.0%)	2/6 (33.3%)	7/16 (43.7%)
≥ 5 years	0/10 (0.0%)	4/6 (66.7%)	4/16 (25.0%)
Gender			
Female	3/10 (30.0%)	3/6 (50.0%)	6/16 (37.5%)
Male	7/10 (70.0%)	3/6 (50.0%)	10/16 (62.5%)
Birth weight (grams)			
< 2,500	1/10 (10.0%)	2/6 (33.3%)	3/16 (18.8%)
≥ 2,500	9/10 (90.0%)	4/6 (66.7%)	13/16 (81.3%)
Length at birth (cm)			
Mean (SD)	48.2 (2.5)	50 (–)*	48.3 (2.4)
Gestational age at birth (weeks)			
< 37	2/10 (20.0%)	2/6 (33.3%)	4/16 (25.0%)
≥ 37	8/10 (80.0%)	4/6 (66.7%)	12/16 (75.0%)
Weight at enrollment (kg)			
Mean (SD)	8.3 (2.5)	29.9 (16.3)	16.9 (14.8)
Length at enrollment (m)			
Mean (SD)	63.7 (12.3)	128.5 (28.2)	88.0 (37.5)
Infant status at enrollment			
Healthy	6/10 (60.0%)	6/6 (100.0%)	12/16 (75.0%)
Not healthy	4/10 (40.0%)	–	4/16 (25.0%)
Disease at enrollment			
Respiratory illness†	2/4 (50.0%)	–	2/4 (50.0%)
Anemia‡	2/4 (50.0%)	–	2/4 (50.0%)

\* Only one case has the data for length at birth.

† Respiratory illness: presence of tachypnea, wheezing, chest retraction, and nasal flaring.

‡ Hb < 10 g/dL.

**Stability.** Stock solutions of BZ in MeOH stored at  $-20^{\circ}\text{C}$  were stable for at least 20 months with a change in measured concentration  $\leq 2.9\%$  from the original preparation. Storage of DBS extracts in the injection buffer (70% MeOH) at room temperature ( $22^{\circ}\text{C}$ ), which simulates the non-temperature-controlled conditions of the autosampler, had little impact ( $\leq \pm 12\%$ DEV) on measured concentrations of BZ, up to 72 hours, as determined in high and low control sample extracts. Long-term storage of DBSs at  $-20^{\circ}\text{C}$  had little effect on BZ measurements: both the low- and middle-concentration controls measured %DEVs  $\leq \pm 0.5$ , 3.8, and 4.2% for storage at 3, 19, and 21 months, respectively.

**Recovery.** The mean percent recovery of BZ from DBSs when spotted at 4,500 and 36 ng/mL was 91.5% and 91.6%, respectively. Overall, a mean percent recovery of 91.5% and a precision (CV%) of 3.6% were observed for the elution methodology.

**Specificity and selectivity.** Method specificity was determined by examining the susceptibility of the assay to interference by biogenic constituents in blank DBSs and

the interference from concomitant medications. There were no observed endogenous peaks that interfered with the quantitation of BZ in DBSs prepared from six unique lots of naive heparinized whole blood (data not shown). Interference from 49 potential concomitant medications, including 21 antiretrovirals, was evaluated by defining the change in signal for the two product ions of BZ ( $m/z = 255$ , the “quantitation” ion and  $m/z = 258$ , or the “qualification” ion) in the presence of compounds co-spotted at a concentration of 10,000 ng/mL. None of the 49 tested compounds produced measurable signals at the retention time of BZ, for either product ion. The QI/Qn ratio was consistent, with no individual concentration %CV exceeding 8.72%, and an overall %CV of  $\leq 5\%$ . Thus, the acceptance criterion for BZ authenticity using this method is a QI/Qn of 0.10,  $\pm 15\%$ .

**Partitioning ratios.** The relative peak areas for the dominant product ion of BZ (Qn), from three different matrices (plasma, DPSs, and DBSs) prepared from fresh whole blood that had been fortified with known amounts of BZ before plasma

 TABLE 2  
 Accuracy and precision

QC	Concentration (ng/mL)	Inter-assay					Intra-assay				
		Mean	Accuracy (%DEV)	SD	Precision (%CV)	n	Mean	Accuracy (%DEV)	SD	Precision (%CV)	n
LLOQ	9.8	10.1	3.5	0.8	7.7	9	10.2	4.4	0.6	6	3
Extra low	12.5	12.2	–2.7	1.5	11.9	9	12.6	0.8	1.7	13.5	3
Low	36	37	2.7	2.6	7.1	9	37.5	4.1	2.2	5.9	3
Middle	180	182.8	1.6	8.2	4.5	9	195	8.3	2.2	1.1	3
High	900	922.6	2.5	44.8	4.9	9	973.3	8.1	46	4.7	3
Extra high	4,500	4,600.5	2.2	160	3.5	9	4,744.5	5.4	76.9	1.6	3

%CV = percent coefficient of variation; %DEV = percent deviation.

TABLE 3  
Partition ratios

	Mean quantitation ion peak areas							All QC
	QC	LLOQ	Extra low	Low	Middle	High	Extra high	
Matrix								
Plasma	–	483.3	587.2	1,794.7	9,405.5	47,018.9	244,404.7	–
DPSs	–	487.6	541.3	1,753.8	9,000.4	43,531.4	235,120.2	–
DBSs	–	475.1	568.7	1,699.4	9,154.0	47,854.8	237,021.0	–
Partition ratios								
DBSs/plasma	–	0.98	0.97	0.95	0.97	1.02	0.97	0.98
DPSs/plasma	–	1.01	0.92	0.98	0.96	0.93	0.96	0.96

DBS = dried blood spot; DPS = dried plasma spot.

generation or card spotting, were evaluated. Qualifier ion acceptance criteria (for QI/Qn) in these partitioning experiments fell within the above acceptance criteria (0.10,  $\pm 15\%$ ). These data demonstrate, within limitations associated with in vitro testing, there is no statistical difference between BZ measurement in all three matrices (plasma, DPSs, and DBSs), for the range of concentrations tested (Table 3).

**Clinical application of the BZ DBS assay.** Sparse pharmacokinetic data were available for 16 participants (65 BZ DBS concentrations). Eight of the 65 (12%) samples had BZ concentrations below the LLOQ of 9.8 ng/mL. Samples below the LLOQ were assigned values equal to one-half of the LLOQ (4.9 ng/mL). The median (IQR) BZ concentration was 1.2 (0.29, 2.14)  $\mu\text{g/mL}$ . Seven of 65 (11%) samples were above the BZ treatment goal of 3  $\mu\text{g/mL}$ . Monte Carlo simulations using the Altchek et al.<sup>13</sup> population model predicts our study population to have median (IQR) values of 2.57 (1.53, 4.48)  $\mu\text{g/mL}$  for troughs, 3.85 (2.73, 5.77)  $\mu\text{g/mL}$  for average steady-state concentrations ( $C_{ss,av}$ ), and 46.25 (32.81, 69.21)  $\mu\text{g} \times \text{hour/mL}$  for  $\text{AUC}_{0-\tau}$  following 5 mg/kg/day BZ divided twice daily. Benznidazole DBS concentrations plotted over the simulated median, 5th, and 95th percentile concentration versus time curves are shown in Figure 1.

## DISCUSSION

An analytical method for the measurement of BZ in human DBSs is required to assess BZ levels of patients enrolled in

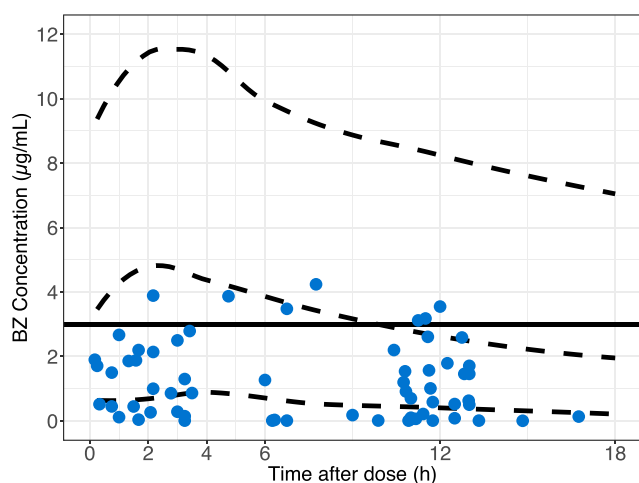


FIGURE 1. Observed and individual predicted benznidazole (BZ) concentrations. The dashed lines display the simulated median, 5th, and 95th percentile concentration versus time curves. The horizontal line displays the target therapeutic trough level (3  $\mu\text{g/mL}$ ). This figure appears in color at [www.ajtmh.org](http://www.ajtmh.org).

Chagas disease clinical trials that are conducted in resource-limited environments where conventional plasma sampling methodologies are impractical. The reported DBS assay provides a simple method to assess therapeutic adherence to BZ in terms of collection, storage, and shipping. The ease of collection allows for the field study personnel to collect blood specimens without compromising the integrity of the sample.

Methods based on ultraviolet detection (UVD) of BZ in plasma after separation by HPLC were deemed unsuitable for the DBS assay owing to their susceptibility to interference from pigments and other components of whole blood. In addition, the vast majority of HPLC–UVD methodologies lack the sensitivity required to accurately quantify BZ in DBS samples, which represent sample volumes equivalent to 5–20  $\mu\text{L}$  of plasma. Our DBS liquid chromatography–tandem mass spectrometry method with enhanced sensitivity (LLOQ = 9.8 ng/mL) is especially suitable for the measurement of BZ from samples obtained well after oral dosing, which can occur when evaluating simple dose adherence. From the in vitro experiments presented here, BZ was shown to partition in an equivalent manner between plasma and whole blood, which strengthens the justification for the use of DBS sampling for monitoring BZ pharmacotherapy.

Additional in vitro characterization of plasma partitioning should be assessed to confirm the validity of DBSs as a surrogate matrix for BZ before using our assay for pharmacotherapeutic monitoring. A more thorough evaluation of potential matrix effects associated with the mass spectrometer–based DBS assay, as per guidelines established by Matuszewski et al.,<sup>24</sup> would fortify this method's validation. In addition, the ULOQ (5,000 ng/mL) is not ideal to capture  $C_{max}$ , particularly in adults. Although none of the 65 BZ observations in our study exceeded the ULOQ, an increase of the ULOQ to 20  $\mu\text{g/mL}$  or more may be warranted to cover the full concentration range for adult and pediatric populations in multiple-dose BZ studies. However, with liquid chromatography–tandem mass spectrometry systems, this will present a challenge, as detector saturation will occur at higher concentrations assuming an LLOQ of 9.8 ng/mL. Thus, there is a trade-off between the desired sensitivity and the ULOQ, if dilution protocols or other methods, such as simultaneous monitoring of  $^{12}\text{C}$ - and  $^{13}\text{C}$ -analyte isotope transitions, are not incorporated into the assay. The concentration range of our BZ DBS assay is best suited for adherence measurements or single-dose pharmacokinetic studies.

In vitro studies have shown that BZ is trypanosomicidal at concentrations ranging from 3 to 6  $\mu\text{g/mL}$ .<sup>25</sup> Recent studies of BZ in pediatric populations suggest that BZ is efficacious

at concentrations markedly lower than adults,<sup>13</sup> which has led to the examination of dosing regimens lower than the standard 5 mg/kg/day dose in adults.<sup>14</sup> At doses ranging from 5 to 8 mg/kg/day, median BZ  $C_{ss,av}$  concentrations were estimated to increase from 4.1  $\mu\text{g/mL}$  for infants aged less than 2 months up to 8.7  $\mu\text{g/mL}$  for children aged 7–12 years.<sup>23</sup> In the same study, median trough concentrations were approximately 2  $\mu\text{g/mL}$  (ages 0–12 years). If adherent, simulations show that our population (median age 12.9 months) would be expected to have a median  $C_{ss,av}$  of 3.85  $\mu\text{g/mL}$  when dosed at 5 mg/kg/day. Whereas only 3/65 (3.5%) BZ DBS specimens were greater than 3.85  $\mu\text{g/mL}$  in the present study, roughly half (32/65) of the specimens were sampled near the trough (10.4–14.8 hours post-dose). Of the 32 specimens collected near the trough, median observed trough concentrations (0.81  $\mu\text{g/mL}$ ) were notably lower than previous reports in a similar population dosed at 5 to 8 mg/kg/day (2  $\mu\text{g/mL}$ )<sup>23</sup> and lower than the median trough estimated in our simulation (2.57  $\mu\text{g/mL}$ ). However, in our study doses were unwitnessed relying on maternal recall for adherence and dosing schedule. With random, sparse, and skewed sampling, a population pharmacokinetic analysis is needed for further characterization of our results. The results of our DBS/plasma partitioning study (mean 0.98) do not support different matrices as the cause for lower than expected BZ concentrations. Reduced absorption, lack of adherence, inaccurate maternal recall, or all may have resulted in markedly lower BZ levels in this population.

The DBS liquid chromatography-tandem mass spectrometry method reported herein is a simple and accurate method for the measurement of BZ within the concentration range of 9.8 and 5,000 ng/mL and is well suited for application to clinical studies and/or monitoring BZ adherence in resource-limited environments.

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**Authors' addresses:** Jeremiah D. Momper, Nathan J. Hanan, Steven S. Rossi, and Edmund V. Capparelli, University of California, San Diego, La Jolla, CA, E-mails: jmomper@ucsd.edu, nhanan@nektar.com, srossi@ucsd.edu, and ecapparelli@ucsd.edu. Mark H. Mirochnick, Boston University School of Medicine, Boston, MA, E-mail: mark.mirochnick@bmc.org. María Luisa Cafferata and Alvaro Ciganda, Unidad de Investigación Clínica Montevideo (UNICEM), Montevideo, Uruguay, E-mails: marialuisa.cafferata@gmail.com and aciganda@unicem-web.org. Antonia Lavenia and Isolina Flores, Ministerio de Salud Pública de la Provincia de Tucumán, Tucumán, Argentina, E-mails: antolavenia@hotmail.com and isoflores@hotmail.com. Luz Gibbons, Instituto de Efectividad Clínica y Sanitaria (IECS), Buenos Aires, Argentina, E-mail: lgibbons@iecs.org.ar. Sergio Sosa Estani, Instituto de Efectividad Clínica y Sanitaria (IECS), Buenos Aires, Argentina, and Instituto Nacional de Parasitología "Dr. Mario Fatała Chaben," CONICET, ANLIS, Buenos Aires, Argentina, E-mail: ssoesaestani@gmail.com. Pierre Buekens, School of Public Health and Tropical Medicine, Tulane University, New Orleans, LA, E-mail: pbuekens@tulane.edu.

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