# Benznidazole levels in blood vary with age in rats

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*Benznidazole (Bz) exhibits toxic side effects in animal studies and clinical use. Reductive metabolism of Bz in liver microsomes modulates the duration of its chemotherapeutic effect and its toxicity. The rate of this metabolism depends on age and is less intense in newborns and youngsters than in adults. In the present study, we determined Bz blood levels in rats of different ages that received Bz intragastrically (100 mg/kg). We developed and validated a high-pressure liquid chromatography with UV detector method for determination of Bz levels in whole blood. Bz levels were significantly higher and persisted for longer periods of time in the blood of young rats when compared to that of adult animals.*

Key words: benznidazole - rat blood - HPLC - Chagas disease

Chagas disease is endemic in Central and South America. Approximately 16-18 million people are infected with *Trypanosoma cruzi* and 100 million are at risk of infection (Pinto Dias et al. 2002, Coura & Castro 2002, Teixeira et al. 2006, Jannin & Villa 2007, Rassi Jr et al. 2009). Two drugs are available for the etiological treatment of Chagas disease: a nitrofurane [nifurtimox (Nfx)] and a nitroimidazole [benznidazole (Bz)] (Tanowitz et al. 1992, Rodriguez-Morales 2005, Jannin & Villa 2007). Currently, Bz is used in Argentina, Brazil and Uruguay to treat the acute or indeterminate chronic phases of Chagas disease (Pinto Dias et al. 2002, Jannin & Villa 2007, Sosa-Estani et al. 2009, Boiani et al. 2010). These drugs are widely accepted for treatment of the acute phase of the disease (OPS/OMS 1998, Coura & Castro 2002, Rodriguez Morales 2005, Jannin & Villa 2007). However, their use in the treatment of the chronic phases of the disease remains controversial (Rodriguez Morales 2005, Jannin & Villa 2007, Castro et al. 2008). Most acutely infected patients are children with a mean age of four years. Congenital transmission to newborns contributes to the young average age of patients, in addition to the triatomine vectors. The congenital route of infection might be relevant to endemic areas as well as non-endemic areas in future years as infected individuals migrate later in life. Public health policies have been implemented in endemic areas, with the aims of identifying pregnant women positive for *T. cruzi* as well as promptly treating infected children immediately after parasitological and serological confirmation (Pinto Dias et al. 2002, Coura & Castro 2002, Sosa Estani et al. 2009). It is important to emphasise that both the duration of Bz chemotherapeutic effects as well as its toxicity are fundamentally controlled by its reductive metabolism in many organs and the faeces. The available information on Bz metabolic transformations and the mechanism of toxicity was exhaustively detailed in previous reviews (Castro & de Toranzo 1988, Castro 2000, Castro et al. 2006). A major part of Bz metabolism in the rat body occurs in the liver microsomal fraction (Masana et al. 1984). Enzymatic activity is present at very low levels in newborn rats and higher levels in 28-day-old rats (Aguilar et al. 1987). These findings spurred us to investigate the blood levels of Bz at different times after intragastric administration, which might vary after administration based upon the age of the patient. To begin, we needed a validated high-pressure liquid chromatography with UV detector (HPLC-UV) methodology for the determination of Bz levels in whole blood. Outbred male Sprague Dawley rats 14, 21 and 70-days-old weighing 29-32 g, 43-46 g and 260-290 g, respectively, were used (5 animals per group). Animals were kept in a controlled room with a 12 h light: 12 h dark cycle (light phase from 6-18 h), temperature of 23 ± 2ºC and relative humidity of 45-65%. The procedures used for breeding, housing and handling animals were those described by the National Food, Drug and Medical Technology Administration (Buenos Aires). Animals had free access to food and water. N-benzyl-2-nitro-1-imidazole acetamide (Bz, 98.6% pure) was a gift from Roche. Animals were given a single Bz dose of 100 mg/kg bw as a homogeneous suspension in 1% carboxymethylcellulose (CMC), while control rats received only 1% CMC. Animals were euthanized by decapitation at different times (1, 3, 6 and 24 h) post-treatment, and blood samples were taken in heparinised tubes and processed. One millilitre of blood sample was diluted with 1.5 mL of saline solution. The samples were extracted with dichloromethane (15 mL) in an Extrelut® column (prepared in 10 mL syringe with 2.4 g of Extrelut®, Merck Chemical). Solvent was evaporated to dryness under a nitrogen atmosphere. Samples were resuspended in 0.5 mL of mobile phase ($CH_3OH$:$H_2O$, 60:40), filtered with nylon filters (pore

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size 0.22 µm) and analysed (50 µL) by HPLC using a Waters system with a $C_{18}$ ODS Hypersil column (20 cm x 2.1 mm ID, 5 µm particle size) and a diode array detector (DAD). The mobile phase, consisting of $CH_3OH$:$H_2O$ (60:40), was delivered isocratically at a flow rate of 0.2 mL/min. Column effluent was monitored at 320 nm (maximum absorbance of Bz). Bz was quantified by the peak-area ratio compared to a standard calibration curve which was made with identically-treated standards ranging from 0.5-100 µg/mL. A standard solution of Bz (100 µg/mL) in $CH_3OH$:$H_2O$ (60:40) was prepared from a stock solution of Bz (1 mg/mL of dimethylformamide) and stored in glass vials at -20ºC (it was stable more than one month). USP 30-NF 25, ICH Q2B and FDA Bioanalytical guidelines were used for validation of the method. The main parameters assayed were: accuracy, precision, specificity, linearity, detection limit and robustness.

*Accuracy* - Blood samples were spiked with known Bz concentrations (1.5, 5, 15, 30 and 75 µg/mL). After extraction of the analyte from the matrix and injection into the HPLC system, its recovery was determined by comparing the response of the extract with the response of the standard (identical spiked Bz concentration in physiologic solution). The recovery efficiency of Bz added to blood was 94%. The detection limit was 0.25 µg/mL, based on the signal to noise approach (ratio of 3:1).

*Precision* - The precision of the Bz assay was determined by repeatability (intraday) and intermediate precision or reproducibility (interday) in samples containing 10 µg/mL of Bz. The results were expressed as the relative standard deviation of a series of measurements.

*Repeatability* - Obtained by examining 10 duplicated, separated samples and analysing each one three times by HPLC.

*Reproducibility* - Determined through the use of different analysts on successive days. We used three samples for triplicate and analysed each one three times by HPLC.

*Specificity* - Not applicable. The absence of matrix interferences for the quantitative method was demonstrated by the analysis of five control animals in each assay.

*Robustness* - In this test, we examined the capacity of the assay to be affected by small but deliberate variations in procedural parameters such as temperature (25ºC and -20ºC) and light conditions (light or darkness). The significance of differences between mean values was assessed by the unpaired t test (Student *t* test) (Gad 2001). Calculations were performed using GraphPad Software. Differences were considered significant when $p < 0.05$. A characteristic chromatogram of a blood sample from an animal treated with Bz showed a mean retention time of 3.7 ± 0.2 min, with an overall chromatographic run time of 6 min. There were no chromatographic interferences; the biological matrix didn't interfere with drug determination. The peak area of Bz vs. its concentration displayed a linear relationship over the tested range of 0.5-100 µg/mL (y = 370253x - 44530). The linear regression analysis indicated no significant deviation from linearity ($r^2$: 0.9995). Replicate analysis of blood samples containing Bz gave an intra-day coefficient of variation of 4.35% (Table I). Analysis of the samples on different days yielded an interday coefficient of variation less than 5% (Table I). The robustness assay showed that the method is robust for small variations in parameters such as temperature (25ºC and -20ºC) and light conditions (light or darkness) (Table I). The initial part of our work was the development and validation of an accessible HPLC-UV methodology for the determination of Bz levels in rat whole blood. The procedure, in our hands, have to be accurate, reproducible, repetitive and robust (Table I). The procedure might be applicable to human-derived samples. That possibility remains to be investigated, considering that other drugs or xenobiotics resulting from additional treatments or exposures may be simultaneously present in human-derived samples. The above methodology was used to determine whole blood levels at times ranging from 1-24 h in 14, 21 and 70-day-old rats that received a single dose of 100 mg/kg of the drug. At 14-days-old, rats have 61% of the liver microsomal capacity to metabolise Bz compared to the adult rat and at 21-28-days-old, the level of Bz-nitroreductase activity attains the level of that of an adult animal (Aguilar et al. 1987). Accordingly, whole blood levels of Bz at 1, 3, 6 and 24 h after administration in the 14-day-old animals are significantly higher and last longer than in those of 21 or 70-day-old rats (Table II). These re-

TABLE I

Validation parameters of benznidazole (Bz) high-pressure liquid chromatography with UV detector (HPLC-UV) assay in rat blood samples

| | Bz (µg/mL) | |
|---|---|---|
| | Mean ± SD | RSD |
| Accuracy (n = 9) | 8.14 ± 0.34 | 4.18 |
| Precision | | |
| Repetitivity (n = 20) | 7.93 ± 0.35 | 4.35 |
| Reproducibility | | |
| Analyst I, day 1 (n = 9) | 7.96 ± 0.39 | 4.89 |
| Analyst I, day 2 (n =9 ) | 8.03 ± 0.34 | 4.24 |
| Analyst II, day 1 (n = 9) | 7.89 ± 0.32 | 4.07 |
| Robustness (n = 9) | | |
| 25ºC light | 7.01 ± 0.35 | 5 |
| 25ºC darkness | 7.56 ± 0.32[a] | 4.25 |
| -20ºC | 7.57 ± 0.34[b] | 4.41 |

*a*: 25ºC light vs. 25ºC darkness: $p > 0.05$; *b*: 25ºC light vs. -20ºC: $p > 0.05$. RSD: relative standard deviation. Repeatability was obtained examining 10 duplicated separated samples analysed three times each one by HPLC system. Reproducibility was determinate with different analysts in successive days. We used three samples for triplicate and analysed three times each one by HPLC system. Analyst I, day 1 vs. analyst I, day 2: $p > 0.05$. Analyst I, day 1 vs. analyst II, day 1: $p > 0.05$. In robustness, values represent means ± SD from nine independent samples analysed three times by HPLC.

TABLE II

Benznidazole (Bz) content in rat whole blood samples

| | Bz concentration (μg/mL blood) | | | |
|---|---|---|---|---|
| | Treatment time (h) | | | |
| Age | 1 | 3 | 6 | 24 |
| Young | | | | |
| 14 days | 37.41 ± 11.35 | 66.04 ± 17.36 | 66.00 ± 18.46 | 8.27 ± 2.06 |
| 21 days | 26.6 ± 9.88 | 64.59 ± 29.54 | 40.82 ± 13.45 | ND |
| Adults | | | | |
| 70 days | 21.74 ± 3.4[a] | 43.21 ± 6.57[a] | 38.92 ± 9.75[a] | 1.58 ± 0.32[a] |

*a*: $p < 0.05$ (adults vs. 14 days old rats). Animals were given a single intragastric dose of 100 mg Bz/kg body weight, as a homogeneous suspension in 1% carboxymethylcellulose (CMC); control received 1% CMC. Animals were euthanized by decapitation at different times (1, 3, 6 and 24 h) and blood samples were taken and processed. Values represent means ± standard deviation from five independent determinations. ND: not detected.

sults underscore the relevance of liver microsomal Bz nitroreductase enzymes in controlling blood levels of this nitroheterocyclic drug. Those enzymes' activities are particularly low in newborn animals (e.g. 1-7-day-old rats) (Aguilar et al. 1987). P450 reductase and P450 enzymes, to a smaller extent, mediate nitroreduction of Bz in rat liver microsomes (Masana et al. 1984). Both enzymes have long been known to be deficient perinatally in liver microsomes from most species of laboratory animals, as well as from humans P450 and P450 reductase mixed-function oxygenase activity in human livers is particularly low in newborns and remains lower than activity in adults during the first four years of life. An additional source of relevant anaerobic metabolic degradation of Bz is present in human faeces (Toranzo et al. 1983). Newborns lack bacterial faecal ability to biotransform xenobiotics (Scheline 1973). This study and past results (Aguilar et al. 1987) provide a rationale for the observation that Bz treatment of young children is accompanied with fewer undesirable side effects than treatment of adults (Pinto Dias 2000, 2003). Bz nitroreductive metabolism controls pharmacokinetic properties, the deleterious effects on different tissues (Castro et al. 2006) and antiparasitic effects (Docampo & Moreno 1986) due to the generation of reactive intermediates during the reductive process that covalently bind to the nuclear and kinetoplastic DNA, proteins and lipids of *T. cruzi* (de Toranzo et al. 1988). The activities of the drug metabolising enzymes governing these processes are influenced by multiple factors, including not only age, but also P450 enzyme polymorphisms, hormone status (sex, adrenal, thyroid and insulin), nutritional status (deficiencies and diet), disease and exposure to xenobiotics (drugs, occupational and environmental factors of inhibitory or inductive effects) (Gram & Gilette 1971, Ingelman-Sundberg 2004, Wauthier et al. 2007). Any or all factors may contribute to the variation in the adverse effects of Bz on patients (Castro & de Toranzo 1988, Castro et al. 2006). The efficacy of chemotherapeutic effects of Bz on *T. cruzi* is not only due to available blood levels of Bz or to the metabolic production of reactive metabolites in the parasite itself. Chemotherapeutic activity of Bz against different *T. cruzi* strains is most likely due to differences in drug susceptibility arising from the above-mentioned Bz metabolic activation processes. This variation could account for some contradictory observations concerning the efficiency of Bz treatment in children when studies were performed in different geographic areas. However, Bz pharmacokinetics are more likely to be affected by the differences observed regarding parasitological cure rates between acute and indeterminate (< 15 years old) forms and the later chronic stage [reviewed by Urbina (2009) and Cançado (2002)].

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