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Comparative bioavailability of three benznidazole formulations in healthy individuals: a randomised study

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BACKGROUND Benznidazole (BZN) has been used for more than fifty years in the treatment of Chagas disease (CD). It is produced by only three pharmaceutical companies worldwide: Lafepe (Brazil); Elea (Argentina) and Liconsa (Spain). The therapeutic interchangeability among these products has never been evaluated.

OBJECTIVES To assess bioequivalence between three 100 mg BZN formulations.

METHODS Pharmaceutical equivalence, with dissolution testing, was assessed prior to the bioequivalence study. For bioequivalence, healthy adult participants received 100 mg of BZN formulations after meal, in a randomised clinical trial. Blood BZN concentrations were measured. Pharmacokinetic parameters were determined by non-compartmental analysis.

FINDINGS The BZN Liconsa demonstrated faster *in vitro* dissolution. However, bioavailability was not different between formulations. The mean area under the curve $(AUC)_{84h}$ values were 49431.22 h*ng/mL (SD = 9938.53) to Lafepe, 48974.38 h*ng/mL (SD = 10304.67) to Elea and 48204.17 h*ng/mL (SD = 9342.18) to Liconsa. The mean C_{max} values were 2339.23 ng/mL (SD = 445.53) for Lafepe, 2209.04 ng/mL (SD = 448.02) for Elea and 2303.90 ng/mL (SD = 431.41) for Liconsa. The mean t_{max} was not different between formulations. AUC were higher in women for Elea (20%) and Lafepe (27%). The adverse events did not differ between sexes.

MAIN CONCLUSIONS The 100 mg BZN formulations demonstrated bioequivalence.

Key words: benznidazole - bioequivalence - bioavailability

Discovered in 1909 by Brazilian scientist Carlos Chagas, Chagas disease (CD) is a neglected tropical disease caused by the flagellated protozoan *Trypanosoma cruzi*. It is endemic in 21 Latin American countries and carries significant health and socioeconomic burdens. Although previously confined to vulnerable, mainly rural endemic areas, CD has progressively spread beyond the Americas, with cases now reported on five continents.[1,2] An estimated 8 million people worldwide are infected, and approximately 100 million people are at risk of infection. However, these numbers are likely underestimated, as less than 10% of infected individuals are diagnosed, and approximately 1% has received etiological treatment.[1,3]

There is currently no vaccine for CD, and treatment still relies on two medications that were licensed over 50 years ago: nifurtimox (NFX), launched by Bayer in 1965; and benznidazole (BZN), introduced by Roche in 1971.[4,5] Comparative data on BZN and NFX remain inconclusive in terms of efficacy. Both BZN and NFX are recommended as first-line therapies for CD by Pan American Health Organization (PAHO) / World Health Organisation (WHO) guidelines and may be used interchangeably in terms of therapeutic efficacy.[6] However, BZN is more frequently used due to its simpler dosing regimen and potentially better tolerability regarding adverse events.[7,8]

BZN (N-benzyl-2-nitro-1-imidazole acetamide) is a 2-nitroimidazole derivative with antiprotozoal activity. It is active against multiple life-cycle stages of *T. cruzi*, including intracellular amastigotes, although susceptibility may vary among parasite genotypes and dormant, non-replicative forms may show reduced sensitivity. In the acute phase of CD or during its reactivation, BZN demonstrates an effectiveness rate of 65-80%, with a significant chance of cure. Some studies also suggest

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efficacy in chronic phase, except in advanced chronic Chagas heart disease, where BZN significantly reduced parasitaemia, but it did not translate into improvement in the evaluated clinical outcomes.[9,10,11,12,13]

Understanding the pharmacokinetic (PK) profile of BZN is essential for optimising treatment regimens in patients with CD. For over four decades, PK data were based on formulations that are no longer available, such as Rochagan® and Radanil®, previously produced by Hoffmann-La Roche. In 2003, BZN production was transferred to the Laboratório Farmacêutico do Estado de Pernambuco Governador Miguel Arraes (Lafepe), a government-affiliated Brazilian manufacturer, initially using the active pharmaceutical ingredient (API) donated by Roche. Since 2011, Lafepe has sourced its API from Nortec Química (Brazil).[14]

In Argentina, production began in 2012 through the private Laboratorio Elea Phoenix SA (Abarax®), in collaboration with the Ministry of Health and the Non-Governmental Organisation Fundación Mundo Sano, using API from Maprimed Farmoquimico Laboratory (Argentina). By 2014, Elea partnered with Liconsa SA (Chemo Group, Spain), resulting in a formulation that received Food and Drug Administration (FDA) approval in 2017 for paediatric use (ages 2-12 years). The FDA registration identifies Empresa Quimica Sintética (Spain), also part of the Chemo Group, as the API supplier for Liconsa. Currently, three manufacturers produce BZN, each relying on different API suppliers.[14]

Liconsa has been registered with the FDA as a reference standard for the 100 mg BZN dose since August 2017. Lafepe obtained reference product status in Brazilian market in 2024 (one year after this study), and Elea is authorised in Argentina. These three products occupy a unique position in their respective markets, with no registered generic or similar alternatives.[14] However, the lack of established bioequivalence among them limits therapeutic interchangeability, restricts patient access to therapy, and difficulties the interpretation and generalisation of clinical study results involving these formulations.

More recently, pharmacokinetic studies using currently marketed formulations from Lafepe and Elea have emerged,[15,16,17] although without explicit bioequivalence assessments. The present study provides novel data by directly comparing the pharmacokinetic profiles and bioequivalence of three currently available 100 mg BZN formulations (Lafepe, Elea, and Liconsa) under controlled conditions in healthy subjects.

This study aimed to compare the bioavailability of 100 mg BZN tablets manufactured by Lafepe, Elea, and Liconsa following a single oral dose administrated to healthy individuals after a standardised meal. Additionally, the influence of sex on pharmacokinetic parameters and bioequivalence were evaluated.

MATERIALS AND METHODS

Pharmaceutical equivalence - Prior to the bioequivalence study, pharmaceutical equivalence was assessed through in vitro tests, including physicochemical and dissolution evaluations. Three drug formulations were analysed: Benznidazole Lafepe, manufactured by Lafepe (batch 21030012); Abarax, manufactured by Elea Phoenix SA (batch 3218); and Benznidazole Chemo, manufactured by Liconsa SA (batch LC60657).

Every pharmaceutical equivalence study includes quality control tests and a dissolution profile to ensure drug compliance before proceeding to bioequivalence testing. In this study, assessments were conducted based on the monograph of Benznidazole Lafepe. It is important to note that no monograph exists in the pharmacopoeias recommended by the Brazilian Health Regulatory Agency (Anvisa).

The tests performed included evaluation of appearance, identification, average weight, individual weight variation, friability, hardness, disintegration, assay, content uniformity, related substances, dissolution, and, finally, the comparative dissolution profile.

For the dissolution test, a Distek dissolution tester system (Model 6100 Evolution) equipped with USP Apparatus 2 was used. The test was performed in 1000 mL of 0.1 M hydrochloric acid (HCl) at 37ºC, using the paddle method at a stirring rate of 100 rpm. Samples were collected at 10, 15, 30, 60, 90, 120, and 240 min. For analysis, samples were diluted 1:5 in the dissolution medium. The extend of drug dissolution was assessed using a UV-VIS spectrophotometer (Mettler Toledo, Model UV 5) at an optimal wavelength of 324 nm. These physicochemical tests adhered to the parameters specified in Brazilian regulatory guidelines.[18]

Bioequivalence study - The *in vivo* study was a single-centre, open-label, randomised crossover trial using three drug formulations of 100 mg BZN tablets: Lafepe, designated as the test formulation (T); Elea, designated as comparator formulation one (R1); and Liconsa, designated as comparator formulation two (R2). The same batches evaluated in the pharmaceutical equivalence test were used. Treatments were administered as a single 100 mg dose in three periods, according to a randomised sequences of treatment (R1TR2, R2R1T, TR2R1), following a Latin Square design. The randomisation list was generated using the PROC PLAN procedure in SAS® software version 9.4. A seven-day washout period was implemented between treatments.

The bioanalytical analyses were conducted in a laboratory certified by Anvisa, in compliance with Brazilian regulatory requirements for bioanalytical studies.[19] All analytical equipment used in the study undergoes routine qualification, calibration, and preventive maintenance in accordance with the laboratory's quality management system and applicable regulatory standards.

Study population, blood samples and adverse events monitoring - Healthy male and female participants aged 18 to 50 years were recruited from a single clinical research centre to ensure standardised study conditions and minimise operational variability. Baseline laboratory tests [renal and hepatic function, lipid profile, complete blood count, and viral markers for hepatitis B and C and anti-human immunodeficiency virus (HIV)] were performed to confirm participant eligibility (healthy condition). Exclusion criteria included liver or gastrointestinal diseases that could affect BZN absorption or

metabolism, pregnancy, breastfeeding, smoking, and a history of alcohol or drug abuse. Use of any other medications was prohibited for two weeks before the study, except for dipyrone, paracetamol, and contraceptives. Participants were also required to abstain from alcohol for 48 h before the study, and were instructed to follow standard dietary restrictions, including overnight fasting prior to drug administration, as commonly applied in pharmacokinetic studies.

Participants underwent three confinement periods of 36 h each, with a seven-day interval between them. In each period, BZN tablets were administered together 200 mL of water, 30 min after a standardised high-fat, high-calorie meal (872.4 calories, with 53.0% fat), which was consumed following a minimum fasting period of 10 h, in accordance with regulatory recommendations for bioequivalence studies conducted under fed conditions to evaluate potential food effects on drug absorption.[20,21]

Blood samples were collected from each participant 30 min before BZN administration (time 0.0 h, baseline), and at 25 time points post-dose: 0.5; 1.0; 1.5; 2.0; 2.5; 3.0; 3.5; 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 10.0; 12.0; 14.0; 24.0; 36.0; 48.0; 60.0; 72.0; and 84.0 h. Blood samples were collected in tubes containing EDTA K3 anticoagulant and centrifuged at 3000 rpm for 10 min at 4ºC (± 2ºC) to separate the plasma fraction, which was immediately stored in an ultra-freezer at -70ºC (± 15ºC) until analysis.

Following each BZN administration, participants were clinically observed for 24 h at the clinical research unit, with monitoring performed by trained medical staff, including vital signs assessment, protocol-specified laboratory evaluations, and monitoring for adverse reactions.

Determination of BZN in blood plasma - Regulatory agency (Anvisa) recommend the analysis of unchanged BZN in bioequivalence studies.[20] Accordingly, BZN was extracted from plasma using a liquid-liquid extraction method with methyl tert-butyl ether (MTBE). For each 100.00 µL of plasma, 50.00 µL of an internal standard working solution [metronidazole (MTZ), 1.00 µg/mL] and 1000.00 µL of MTBE were added. The samples were mixed using an automatic shaker for 1 min and centrifuged for 5 min at 14400 rpm and 10ºC (± 2ºC). Subsequently, 600.00 µL of the supernatant (organic phase) was transferred to a polypropylene microtube, evaporated under a stream of nitrogen gas, and reconstituted with 300.00 µL of dilution solution composed of methanol:acetonitrile (80:20) with 0.10% formic acid in Type I water (70:30 v/v). The samples were mixed at 1500 rpm for 1 minute, and 300.00 µL aliquots were transferred to analysis vials.

BZN quantification was performed using mass spectrometry (API 4000™ AB SCIEX) in positive electrospray ionisation mode (ESI+), combining high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS). The transitions monitored were 261.092 → 91.100 m/z for BZN and 172.099 → 127.975 m/z for MTZ. The BZN calibration curve was linear from 25.00 to 4000.00 ng/mL, with MTZ internal standard maintained at a fixed concentration of 500.00 ng/mL. An ACE 5 µm C18 (150 mm x 4.6 mm) analytical column was used for chromatographic separation.

The analytical method was developed and validated in accordance with Brazilian regulatory guideline.[22]

Pharmacokinetic parameters - Pharmacokinetic parameters were determined by non-compartmental analysis (NCA). The maximum plasma concentration (C_{max}) and time to reach C_{max} (t_{max}) were obtained directly from the individual plasma concentration-time curve. The area under the plasma concentration-time curve from time zero to the last measurable concentration within 84 h [area under the curve $(AUC)_{84h}$] was calculated using the linear trapezoidal method. The total AUC from time zero to infinity (AUC_{∞}) was calculated as $AUC_t + (C_t/\lambda)$, where C_t is the last quantifiable concentration and λ is the apparent terminal elimination rate constant. The plasma elimination half-life ($t_{1/2}$) was calculated as $\ln(2)/\lambda$. Apparent total body clearance (CL) and the apparent volume of distribution in the terminal phase (V_z) were calculated using the equations $CL = Dose/AUC_{\infty}$ and $V_z = Dose/(\lambda * AUC_{\infty})$, respectively, expressed in relation to the bioavailability factor (F) as CL/F and V_z/F.

Pharmacokinetic parameters were determined for each participant and formulation. To evaluate sex-related differences, the nominal administered dose (100 mg) was normalised by each participant's body weight. The weight was measured at each study period. The resulting weight-adjusted dose (mg/kg) was used to adjust individual pharmacokinetic parameters. All analyses were conducted using Phoenix WinNonlin® version 8.4 (Certara).

Statistical analysis - Mean plasma concentration versus time curve were plotted to visually compare the three formulations. Descriptive statistics were calculated for each pharmacokinetic parameters and formulation, for bioequivalence test.

Bioequivalence was assessed using average bioequivalence criteria. A linear mixed-effects model was applied to the log-transformed AUC and C_{max} values, including sequence, period and formulation as fixed effects, and subject nested within the sequence as a random effect. Bioequivalence was concluded if the 90% confidence intervals (90% CI) for the geometric mean ratios (T/R1, T/R2, and R1/R2) of the PK parameters AUC and C_{max} were within the acceptance range of 80% to 125% for 90% CI, following regulatory guidance.[20,23,24]

Comparisons between sex and formulations were performed, using the Mann-Whitney Rank Sum test. Differences were considered statistically significant at $p < 0.05$.

Statistical analyses were conducted using validated software packages, including SAS® 9.4 (SAS Institute Inc.), Phoenix WinNonlin® 8.4 (Certara), and SigmaPlot® 14.1 (Systat Software).

Ethical aspects - Pharmaceutical equivalence and bioequivalence analyses were performed in research centres certified by Anvisa. The study was conducted in accordance with Resolution No. 466/2012 of the Brazilian National Health Council[25] and approved by the Institutional Research Ethics Committee (CAAE: 63382222.4.0000.8098; approval document No. 5.670.824; approved on September 28, 2022). The study protocol was registered in the Brazilian Registry of Clinical Trials (ReBEC) under ID: 63382222.4.0000.80981 and is available at: https://ensaiosclinicos.gov.br/rg/RBR-6zfzs4j.

RESULTS

Pharmaceutical equivalence tests - The drug formulations complied with regulatory guidance for all physicochemical tests (Table I). However, the dissolution profile analysis revealed that Lafepe and Liconsa formulations were not pharmaceutically equivalent, with an F_2 value of 42. The Liconsa formulation exhibited a faster dissolution rate, and it was only after 90 min that both formulations showed similar percentages of drug dissolved (Table II). In contrast, the comparison between Elea and Lafepe yielded an F_2 value of 65, meeting the regulatory threshold for pharmaceutical equivalence (Fig. 1).

Bioequivalence analysis - A total of 42 healthy participants residing in Campinas, São Paulo, Brazil, were enrolled in the bioequivalence study, in 2022. Of these, 39 participants completed the study, 17 men and 22 women, as demonstrated in Fig. 2. The overall median was 36 years (IQR 30-42) [37.50 years for women (IQR 30.75-41.25); 36 years for men (IQR 27-42)] and weight median was 69.1 kg (IQR 64.30-83.10) [66.80 kg for women (IQR 61.88-70.65); 83.10 kg for men (IQR 67.30-89.20)]. The mean plasma concentration versus time curve for each drug formulation is presented in Fig. 3, and the average pharmacokinetic parameters are summarised in Table III.

Bioequivalence was assessed by comparing the bioavailability parameters (C_{max} and AUC) among the three drug formulations. The 90% CI for the ratios of geometric means (AUC and C_{max} for T/R1, T/R2, R2/R1) fell within the accepted bioequivalence range of 0.80 to 1.25 (Table IV). Differences in C_{max} and AUC between

TABLE I

Results of pharmaceutical equivalence

Tests	Elea (R1)	Liconsa (R2)	Lafepe (T)
Appearance	White, circular, flat tablet with a bisecting score on one side	White, circular, flat tablet, bisecting scores on both sides and the letter "E" inscribed in each quadrant on one side	White, circular, flat tablet with a bisecting score on one side
Identification	The sample solution of the test drug exhibited absorbance at a wavelength of 324 nm	The sample solution of the test drug exhibited absorbance at a wavelength of 324 nm	The sample solution of the test drug exhibited absorbance at a wavelength of 324 nm
Average weight	299.61 mg	304.26 mg	252.98 mg
Friability	0.04%	0%	0.26%
Hardness	48 N	62 N	93 N
Disintegration	Last tablet: 30s	Last tablet: 2 min and 32 s	Last tablet: 1 min and 03 s
Assay	102.9%	102.1%	104.6%
Content uniformity	VA = 5.7	VA = 3.6	VA = 3.9
Related substances*	Not detected	Not detected	Not detected
Dissolution	Stage E1 60 min: minimum of 90.9%; 120 min: minimum of 95.3%	Stage E1 60 min: minimum of 88.4%; 120 min: minimum of 90.0%	Stage E1 60 min: minimum of 85.3%; 120 min: minimum of 93.2%

*2-Aminoimidazole sulfate; 2-Nitroimidazole (impurity A); N-Benzylchloroacetamide (impurity B).

TABLE II

Dissolution test for 100 mg benznidazole (BZN) tablets from Elea (R1), Liconsa (R2), and Lafepe (T)

	Elea (R1)		Liconsa (R2)		Lafepe (T)	
Time (min)	Q	RSD	Q	RSD	Q	RSD
0	0.00	0.00	0.00	0.00	0.00	0.00
10	53.30	8.34	72.26	1.68	46.32	3.79
15	66.37	7.65	82.65	1.31	58.85	3.15
30	81.52	6.16	90.38	1.27	76.24	2.19
60	91.81	4.94	95.42	1.36	89.18	1.64
90	92.95	4.43	96.41	1.68	93.53	1.40
120	94.67	4.43	96.79	1.14	96.46	1.26
240	94.52	4.24	96.65	1.42	99.01	1.31

Values are described in percentual (%). Q: dose/solubility ratio (% mean); RSD: residual standard deviation (%).

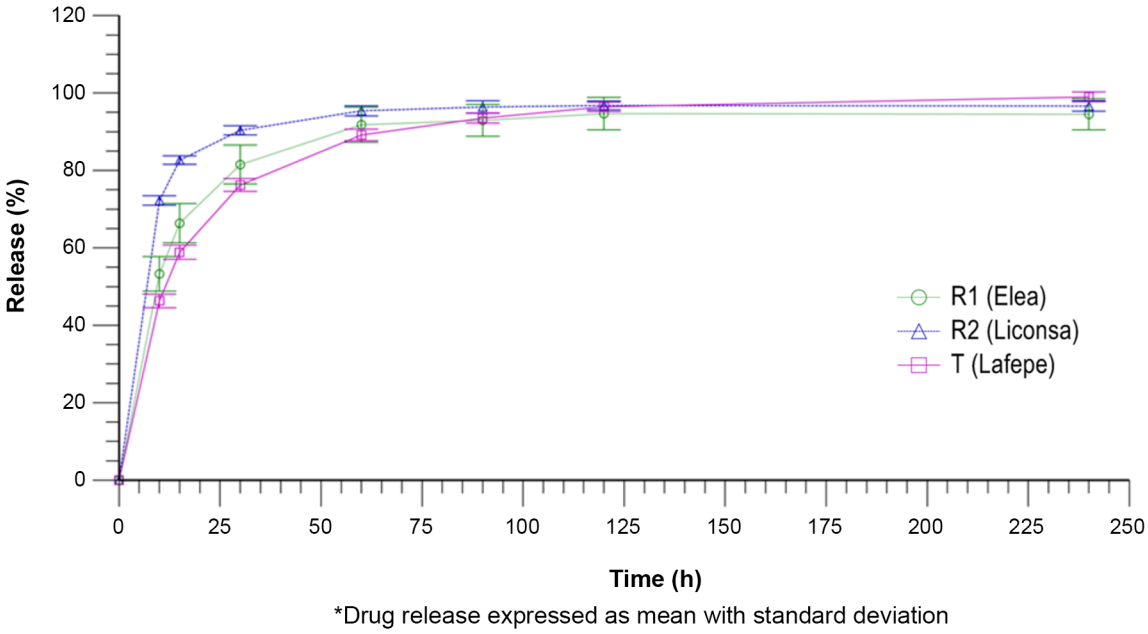


Fig. 1: dissolution profiles of the three benznidazole (BZN) 100 mg formulations obtained during the pharmaceutical equivalence test.

the drug formulations were minimal, not exceeding 6% and 3%, respectively. Intraindividual variability for the pharmacokinetic parameters was low, under 10% for C_{max} and under 7% for AUC. Statistical power exceeded 99,99% for all comparations. The t_{max} was approximately 5 h for all formulations, not differing statistically between formulations.

Distribution (V_z/F) and elimination parameters (CL/F, λ, and $t_{1/2}$) also showed no significant differences among the three formulations, as illustrated in Fig. 4.

Sex-based differences - Plasma concentrations were higher in women for all three formulations (Fig. 5). AUC was higher in women for Elea and Lafepe formulations and C_{max} was higher in women for all (Table V). Female participants had a lower median body weight than male participants (20%), resulting in a more pronounced weight-adjusted dose (mg/kg) in women (25%). Consequently, women could exhibit higher C_{max} and AUC values. After normalising pharmacokinetic parameters by weight (AUC/D and C_{max}/D), no statistically significant differences between sexes were observed. Analysis of covariance (ANCOVA) confirmed that body weight was a significant covariate in the comparison between sexes ($p < 0.001$), influencing BZN exposure. There was no significant interaction between sex and weight for the AUC.

Differences in t_{max} between sexes were observed for the Elea and Liconsa formulations, suggesting a slower absorption rate in women. The $t_{1/2}$ was lower in women for the Liconsa formulation.

Despite its influence on pharmacokinetic absorption parameters (C_{max} and AUC) and apparent difference in impact between the drug formulations studied, sex did not significantly affect the bioequivalence analysis. The linear mixed model for the AUC_{84h} showed no significant sex effect ($p = 0.1030$) or sex-by-formulation interaction ($p = 0.4041$). For C_{max}, a statistically significant sex effect was observed ($p = 0.0058$), but the interaction with formulation was not significant ($p = 0.4690$). The 90% CI for the ratio of geometric mean ratios remained within the regulatory acceptance (80-125%) for all comparisons across sex groups: men [T vs. R1 (103.42; 115.82); T vs. R2 (94.20; 105.49); R2 vs. R1 (103.75; 116.19)]; women [T vs. R1 (99.47; 110.84); T vs. R2 (97.11; 108.21); R2 vs. R1 (97.03; 108.13)].

Security analysis - During the study, a total of 33 adverse events were observed. No serious adverse event was observed, and all reported adverse events were classified as mild (94%) or moderate (6%). Of these events, 21 (64%) were evaluated as having some degree of causality related to the use of BZN. Among them, 11 events (52%) were deemed to have probable or possible causality, consisting of ten cases of headache and one case of vomiting. In a small number of cases, headache was managed with a single oral dose of dipyrone (1 g). No other concomitant medications were required for the management of adverse events. The remaining 10 events (48%) were classified as having conditional causality, which included one case of diarrhoea (3%) and laboratory findings of altered liver enzymes following the treatment periods. There were four events (12%) of elevated gamma-glutamyl transferase (GGT), three (9%) of elevated alanine transaminase (ALT), and two (6%) of elevated aspartate transaminase (AST). These transient increases in liver enzymes were classified as a conditional adverse event due to the absence of clinical symptoms and the isolated nature of the laboratory findings.

Regarding formulation-specific causality, among the 11 adverse events with possible or probable causality: 55% occurred following the administration of formulation R2 (Liconsa), 36% after formulation T (Lafepe), and 9% after

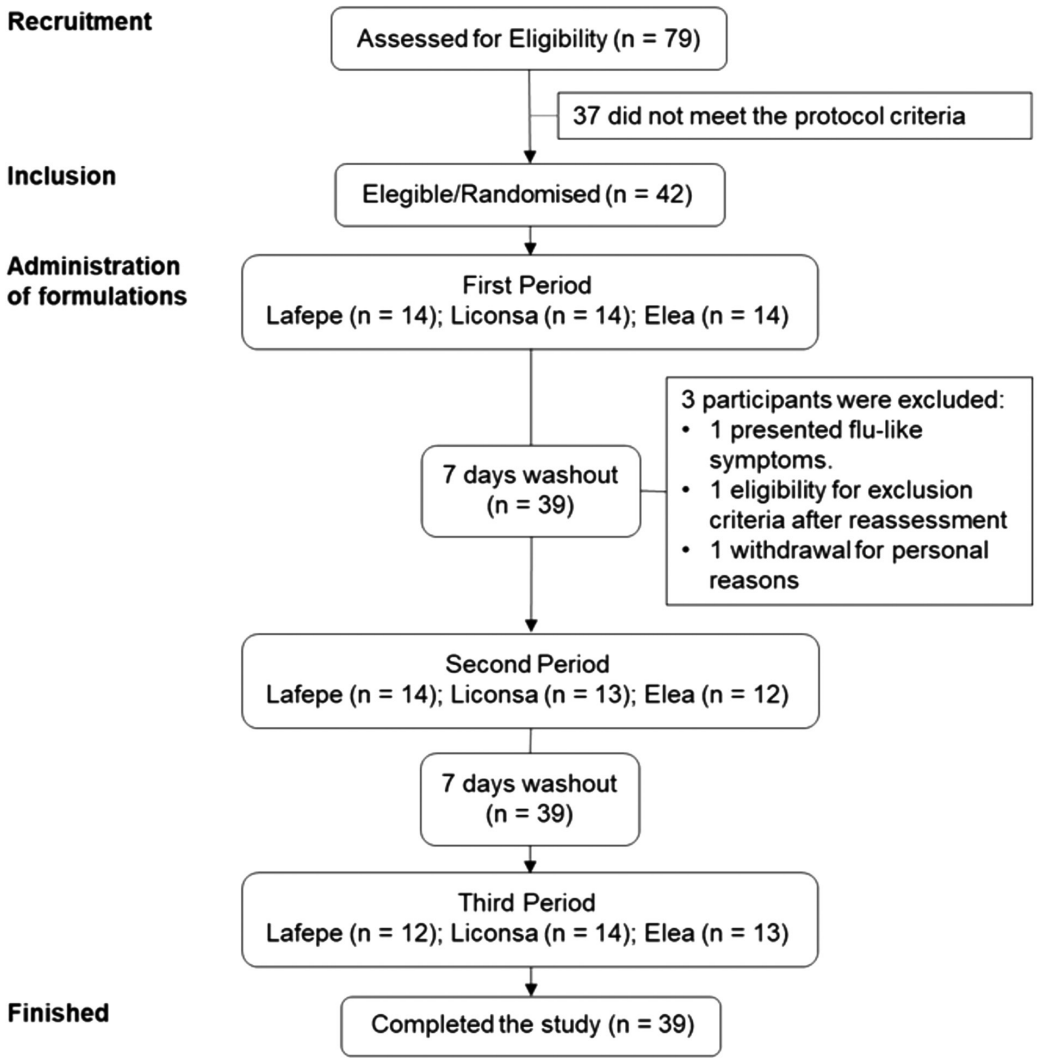


Fig. 2: flowchart to bioequivalence analysis.

formulation R1 (Elea). All adverse events resolved completely, and no long-term effects were observed.

In terms of sex distribution, 60% of the adverse events classified as probable, possible, or conditional causality occurred in women. However, the overall incidence of adverse events between sexes was not statistically significant [relative risk (RR) = 1.23; 95% CI: 0.54-2.80].

DISCUSSION

To our knowledge, this is the first study to compare three currently available BZN formulations, integrating pharmacokinetic evaluation with formal assessments of pharmaceutical equivalence and bioequivalence in healthy participants. For the past 40 years, pharmacokinetic data on BZN has relied on formulations previously produced by Roche, which are no longer available. The most recent formulations, produced by Lafepe (Brazil), Elea (Argentina) and Liconsa (Spain), have not been evaluated for bioequivalence. The lack of published information regarding the interchangeability of these formulations hinders an adequate comparison of results obtained from studies that used different formulations

In vitro tests demonstrated that the three drug formulations showed no significant differences in physicochemical tests and were considered pharmaceutical equivalents. However, dissolution profile analysis showed that the Lafepe and Elea formulations presented equivalent dissolution behaviour, whereas the Liconsa formulation exhibited a different profile, characterised by a faster BZN release. The Liconsa formulation achieved more than 80% drug release within 15 min, compared with approximately 30 min for Elea and 60 min for Lafepe. After 60 min, the unreleased fraction was close to 10% for all three formulations.

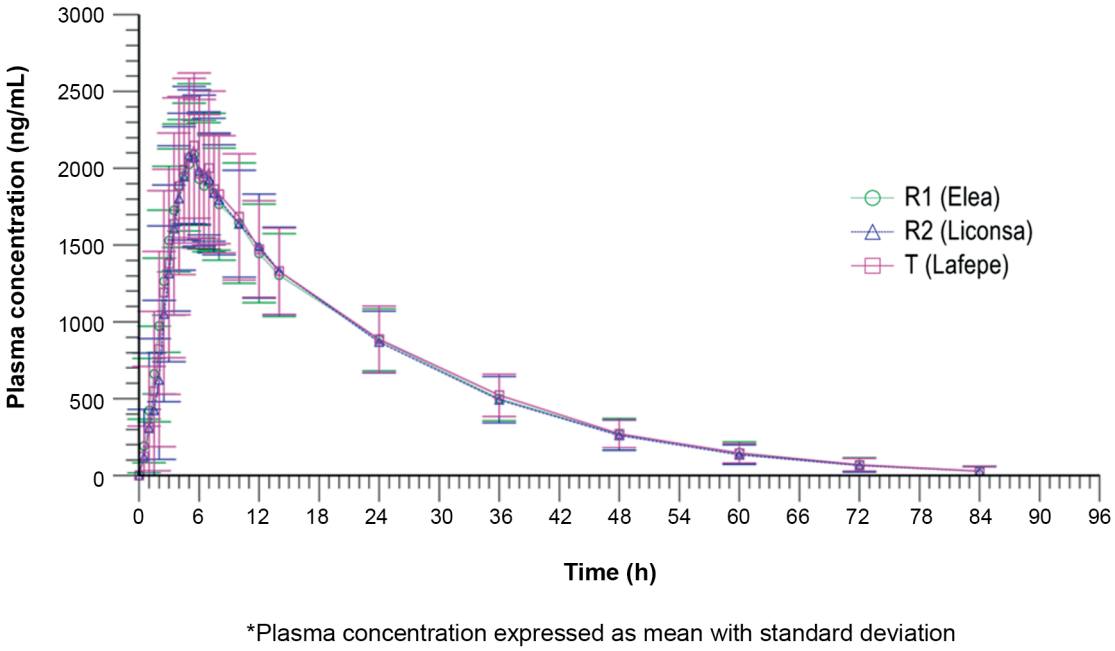


Fig. 3: plasma concentration versus time for 100 mg benznidazole (BZN) tablets from Elea (R1), Liconsa (R2), and Lafepe (T).

TABLE III

Pharmacokinetics parameters for 100 mg benznidazole (BZN) tablets from Elea (R1), Liconsa (R2), and Lafepe (T)

Variable	Units	N	R1 (Elea)	R2 (Liconsa)	T (Lafepe)
t_{max}	(h)	39	4.95 (1.22)	4.71 (1.20)	4.94 (1.42)
C_{max}	(ng/mL)	39	2209.04 (448.02)	2303.9 (431.41)	2339.23 (445.53)
AUC_{84h}	(h*ng/mL)	39	48974.38 (10304.67)	48204.17 (9342.18)	49431.22 (9938.53)
AUC_{∞}	(h*ng/mL)	39	49910.49 (10608.09)	48992.55 (9590.13)	50292.7 (10128.11)
$\%AUC_{extrap}$	(%)	39	1.85 (0.91)	1.59 (0.79)	1.72 (0.82)
λ	(h^{-1})	39	0.0574 (0.0096)	0.0609 (0.0136)	0.0600 (0.012)
$t_{1/2}$	(h)	39	12.45 (2.35)	11.86 (2.36)	11.97 (2.26)
CL/F	(L/h)	39	2.1 (0.49)	2.13 (0.46)	2.08 (0.47)
V_z/F	(L)	39	37.23 (8.91)	35.8 (7.65)	35.61 (9.21)

Values are described as mean (standard deviation). t_{max}: time to reach C_{max}; C_{max}: maximum concentration; AUC_{84h}: area under the curve up to the last measurable concentration within 84 h; AUC_{∞}: area under the curve up to infinity; $\%AUC_{extrap}$: extrapolated percentage of AUC_{∞} in relation to AUC_{84h}; λ: apparent elimination constant; $t_{1/2}$: elimination half-life; CL/F: apparent total body clearance; V_z/F: apparent volume of distribution.

Despite the difference observed in the dissolution test, the products were found to be bioequivalent *in vivo*. The ratios for absorption parameters C_{max} and ASC were close to 1 for all comparisons between formulations (T/R1; T/R2 and R2/R1), and the 90% CI for the geometric mean ratios fell within the regulatory accepted range of twenty percent variation: 0.80 to 1.25.[20,23,24]

The observed discrepancy between the in vitro (dissolution) and *in vivo* (bioequivalence) results is not entirely clear. However, two hypotheses may explain this difference. The first relates to the methodology used in the dissolution test. As already mentioned, a pharmacopeial method has not been established for assessing pharmaceutical equivalence in BZN, and the method used in this study may not have sufficient discriminatory power for these formulations. Furthermore, the exact composition and manufacturing processes of the formulations are not publicly available, which may influence dissolution behaviour. Further evaluation of this hypothesis is warranted.

The second hypothesis, which we consider more relevant, concerns the administration method used during the clinical trial. All three BZN formulations were administered within 30 min after a high-fat, high-calorie meal (872.4 calories, with 53.0% fat). The presence of food can delay gastric emptying, postponing the drug's arrival at the intestinal absorption site.[26,27,28] According to FDA data, administering a 100 mg BZN tablet with

TABLE IV
Bioequivalence analysis for 100 mg benznidazole (BZN) tablets from Elea (R1), Liconsa (R2) and Lafepe (T)

T (Lafepe) X R1 (Elea)					
Parameter	N	Ratio T/R1*	CI 90%**	Power	CV_{intra} (%)***
Ln (AUC_{84h})	39	100.86	(98.43; 103.34)	> 99.99	6.32
Ln (AUC_{∞})	39	100.69	(98.26; 103.18)	> 99.99	6.33
Ln (C_{max})	39	106.72	(102.90; 110.67)	> 99.99	9.44
t_{max} (diference)	39	0.00	(-0.50; 0.50)	-	
T (Lafepe) X R2 (Liconsa)					
Parameter	N	Ratio T/R2*	CI 90%**	Power	CV_{intra} (%)***
Ln (AUC_{84h})	39	102.35	(99.89; 104.88)	> 99.99	6.32
Ln (AUC_{∞})	39	102.47	(100.00; 105.01)	> 99.99	6.33
Ln (C_{max})	39	101.90	(98.26; 105.68)	> 99.99	9.44
t_{max} (diference)	39	0.00	(0.00; 0.50)	-	
R2 (Chemo) X R1 (Elea)					
Parameter	N	Ratio R2/R1*	90% CI**	Power	CV_{intra} (%)***
Ln (AUC_{84h})	39	98.54	(96.17; 100.97)	> 99.99	6.32
Ln (AUC_{∞})	39	98.26	(95.89; 100.69)	> 99.99	6.33
Ln (C_{max})	39	104.72	(100.98; 108.61)	> 99.99	9.44
t_{max} (diference)	39	0.00	(-0.50; 0.00)	-	

*Geometric mean ratio; **Confidence interval of 90%; ***Intra-subject variability (CV_{intra}).

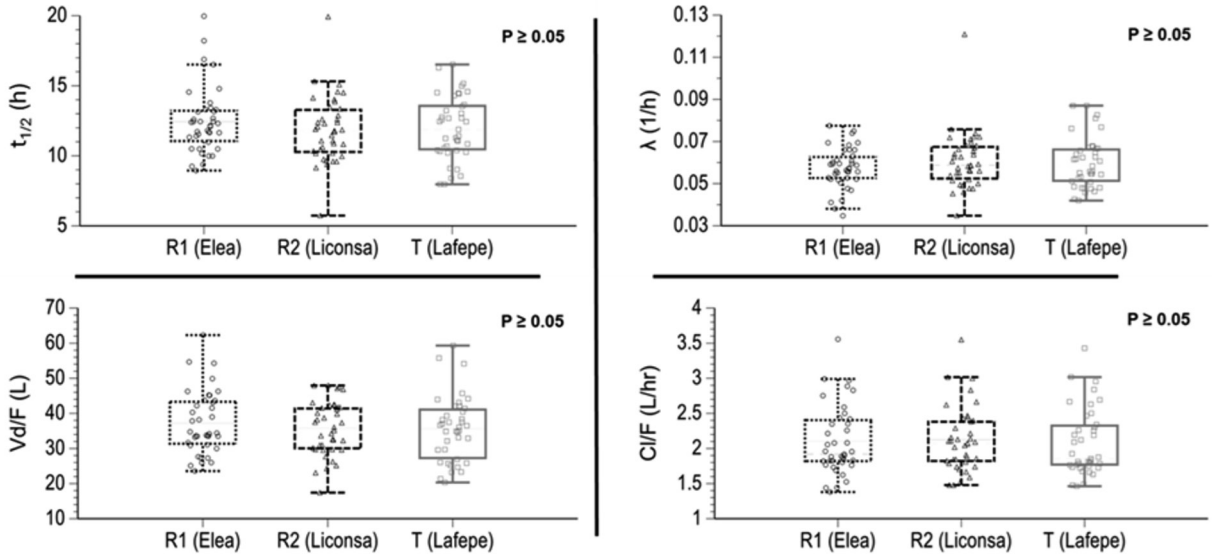
a high-fat, high-calorie meal (approximately 1034 total kcal, 67 kcal from fat, 42 kcal from carbohydrates, 59 kcal from protein) does not alter C_{max} or AUC, but delays t_{max} by approximately 80 min.[29] Similarly, in a study comparing Lafepe BZN pharmacokinetics under feed and fasting conditions, t_{max} was delayed by 90 min with a meal (fed = 5.00 h and fasted = 3.5 h).[15]

The dissolution test simulated the gastric environment, including pH and motility. Within 60 min, 90% or more of the BZN had already been released from all three formulations. Therefore, the delay in drug absorption due to food intake may have masked differences seen in the dissolution test, acting as a rate-limiting step, and equalising the absorption profiles. This finding has important clinical implications. Although food does not significantly affect BZN bioavailability, the drug is typically administered after meals to minimise potential gastric adverse effects (as recommended in the Elea and Lafepe leaflets). As a result, despite differing dissolution rates, the absorption of BZN in the presence of food is likely to be similar across the three formulations in clinical settings.

The pharmacokinetic parameters observed in this trial were similar to those reported in previous studies, after single dose, for bioavailability and elimination parameters,[17,30,31] even those using formulations no longer available on the market.[32] For t_{max}, we observed higher values, which may be explained by the administration of BZN formulations after a high-fat meal. Silveira et al. also suggested the possibility of a higher absorption rate in patients.[17] Additionally, Wiens et al. also found limited variability in BZN pharmacokinetics across published studies between 1979 and 2016.[30]

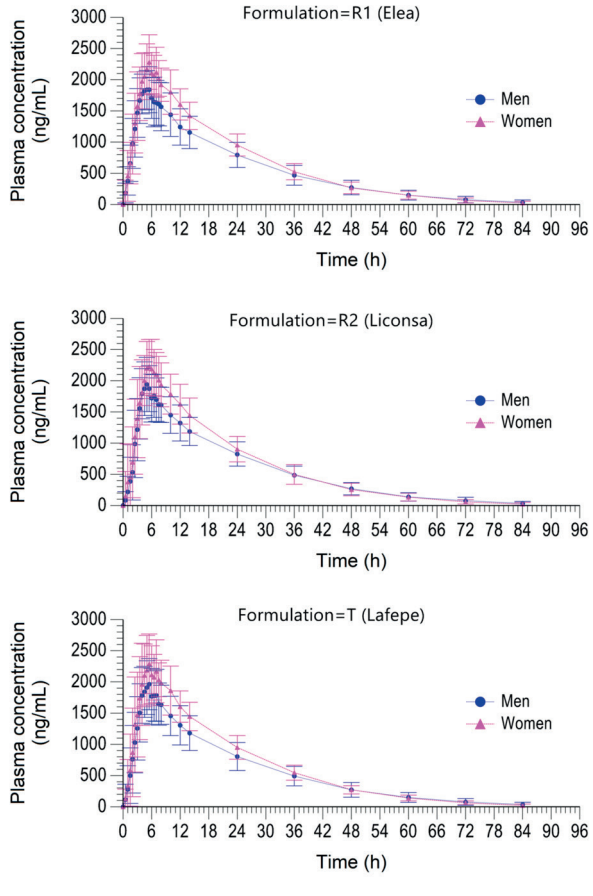
Recently, the bioavailability of two BZN formulations (Lafepe vs. Elea) were compared in 13 adults with chronic *T. cruzi* infection (nine women and four men), after a single 100 mg oral dose under fasting conditions.[16] In agreement with our findings, they reported similar C_{max} and t_{max} values between formulations, although the mean values differed. The t_{max} was 42% lower in the Hernández study, likely due to fasting administration. C_{max} was 28% higher in patients, suggesting potentially increased absorption in this population, a hypothesis requiring further investigation. Supporting this, a higher absorption constant (Ka) was observed in chronically *T. cruzi*-infected Swiss mice compared to healthy controls.[33]

In contrast to our results, Hernández et al. reported greater variability in pharmacokinetic parameters (C_{max} 19.9% vs. 9.44%; AUC 40.7% vs. 6.32%), and shorter $t_{1/2}$ values (8.0 h for Elea and 9.1 h for Lafepe), as well as lower AUC values (32.0 and 37.5 h*ng/mL, respectively for Elea and Lafepe).[16] Differences in blood sampling schedules may account for these differences. In our study, the elimination phase of BZN was best characterised after 24 h in 70% of participants, and after 36 h in 60%. The lack of more samples after 24 h in the Hernández's study (only one sample was obtained) may have led to inaccurate estimates of the elimination phase, using



*P-valor for Mann-Whitney Rank Sum Test. **λ:** R1 x T (P = 0.614), R2 x T (P = 0.642), R1 x R2 (P = 0.345); **$t_{1/2}$:** R1 x T (P = 0.614), R2 x T (P = 0.642), R1 x R2 (P = 0.345); **CL/F:** R1 x T (P = 0.572), R2 x T (P = 0.463), R1 x R2 (P = 0.657); **V_z/F:** R1 x T (P = 0.506), R2 x T (P = 0.693), R1 x R2 (P = 0.664)

Fig. 4: box plot to distribution and elimination pharmacokinetic parameters by drug formulation.



*Plasma concentration expressed as mean with standard deviation

Fig. 5: plasma concentration versus time for Elea (R1), Liconsa (R2) and Lafepe (T) by sex.

data still in the distribution phase, which would have generated a high λ and a $t_{1/2}$ below that commonly found. We observed in our study that only about 24% of the AUC was calculated based on the first 8 h, suggesting that a collection window with fewer points in the distribution and elimination phases (in the Hernández study only three points were obtained after 8 h versus nine points in our study) could have underestimated the AUC and increased the observed variability. However, the possibility of higher variability in patients cannot be ruled out and, as highlighted by Hernández et al., there is still a lack of information regarding factors contributing intra-individual to variability, as well as the influence of population characteristics such as sex, age, and disease status.

Pharmacokinetic data in patients with chronic CD treated with the Lafepe formulation were reported.[17] Although this study was conducted in infected patients under treatment conditions, the exposure parameters observed were broadly consistent with those obtained in the present study following single-dose administration. In agreement with our findings, a variation between sexes was also observed for the pharmacokinetic absorption parameters after administration of fixed doses of BZN.

It is well established that demographic factors like sex can significantly impact drug absorption, distribution and elimination.[34,35] In a study involving eight healthy participants (four women and four men), Molina et al. found that BZN C_{max} was 81% higher in women ($p = 0.02$), and that V_z/F was 42% higher in men ($p = 0.02$) after a single 100 mg dose of Elea BZN.[31]

Our study also assessed the impact of sex on BZN pharmacokinetics for the three formulations. In agreement with Molina and Silveira,[17,31] we found that BZN

TABLE V

Pharmacokinetic parameters by drug formulation and sex

Pharmacokinetic parameter	Formulation	Groups		P**
		Women (N = 22)*	Men (N = 17)*	
C_{max} (ng/mL)	R1 (Elea)	2407.95 [2154.30; 2616.70]	1844.93 [1635.53; 226.14]	< 0.001***
	R2 (Liconsa)	2306.68 [2091.97; 2752.80]	2093.87 [1924.29; 2267.72]	0.016***
	T (Lafepe)	2518.08 [2193.13; 2731.85]	1949.65 [1807.45; 2495.45]	0.002***
C_{max}/D (kg*ng/mL/mg)	R1 (Elea)	1610.71 [1472.60; 1714.71]	1537.63 [1447.77; 1627.51]	0.234
	R2 (Liconsa)	1633.19 [1507.24; 1704.81]	1674.98 [1521.06; 1838.65]	0.322
	T (Lafepe)	1648.19 [1511.68; 1802.63]	1613.87 [1551.67; 1728.95]	0.910
t_{max} (h)	R1 (Elea)	5.50 [4.50; 6.13]	4.50 [3.75; 5.00]	0.007***
	R2 (Liconsa)	5.00 [4.50; 5.50]	4.50 [4.00; 5.00]	0.032***
	T (Lafepe)	5.25 [4.00; 5.50]	4.50 [4.25; 5.50]	0.451
AUC_{84h} (h*ng/mL)	R1 (Elea)	52717.19 [49610.63; 55940.26]	43690.48 [35214.86; 53407.54]	0.023***
	R2 (Liconsa)	52144.60 [45627.48; 55623.98]	45835.82 [37258.63; 50862.47]	0.084
	T (Lafepe)	54324.24 [45127.79; 57207.84]	42785.41 [35974.71; 55130.04]	0.027***
AUC_{84h}/D (kg*h*ng/mL/mg)	R1 (Elea)	35280.60 [31410.69; 37949.33]	33541.27 [29662.07; 39053.13]	0.821
	R2 (Liconsa)	33927.06 [30118.84; 37360.88]	37221.94 [30383.15; 39650.55]	0.365
	T (Lafepe)	36232.02 [32021.38; 39157.72]	34493.03 [30192.09; 38171.90]	0.734
AUC_{∞} (h*ng/mL)	R1 (Elea)	53332.46 [50420.20; 56905.19]	44300.73 [35842.26; 54503.23]	0.025***
	R2 (Liconsa)	52718.79 [46374.63; 56336.82]	46421.73 [37857.33; 51914.27]	0.095
	T (Lafepe)	55368.61 [45685.86; 57991.85]	43423.41 [36621.87; 56114.66]	0.041***
AUC_{∞}/D (kg*h*ng/mL/mg)	R1 (Elea)	35737.87 [37769.48; 38324.88]	34160.19 [30142.16; 39854.66]	0.843
	R2 (Liconsa)	34319.38 [30358.04; 37704.27]	37770.12 [30828.63; 40329.28]	0.308
	T (Lafepe)	36671.12 [32369.49; 39555.41]	35418.02 [30685.43; 38967.35]	0.799
$\%AUC_{extrap}$ (%)	R1 (Elea)	1.32 [1.19; 1.88]	1.81 [1.44; 2.86]	0.010***
	R2 (Liconsa)	1.29 [0.87; 1.65]	1.67 [1.36; 2.06]	0.018***
	T (Lafepe)	1.31 [0.89; 1.89]	1.64 [1.47; 2.61]	0.022***
λ (1/h)	R1 (Elea)	0.0590 [0.0542; 0.0666]	0.0557 [0.0473; 0.0595]	0.070
	R2 (Liconsa)	0.0633 [0.0563; 0.0685]	0.0551 [0.0477; 0.0632]	0.022***
	T (Lafepe)	0.0621 [0.0540; 0.0673]	0.0546 [0.0480; 0.0640]	0.113
$t_{1/2}$ (h)	R1 (Elea)	11.75 [10.41; 12.79]	12.45 [11.65; 14.67]	0.070
	R2 (Liconsa)	10.94 [10.12; 12.32]	12.59 [10.97; 14.54]	0.023***
	T (Lafepe)	11.15 [10.29; 12.83]	12.69 [10.83; 14.45]	0.113
CL/F (L/h)	R1 (Elea)	1.88 [1.76; 1.98]	2.26 [1.83; 2.79]	0.025***
	R2 (Liconsa)	1.90 [1.78; 2.16]	2.15 [1.93; 2.64]	0.095
	T (Lafepe)	1.81 [1.72; 2.19]	2.30 [1.78; 2.73]	0.039***
CL/F II (L/h/kg)	R1 (Elea)	0.0280 [0.0261; 0.0315]	0.0293 [0.0251; 0.0332]	0.843
	R2 (Liconsa)	0.0291 [0.0265; 0.0329]	0.0265 [0.0248; 0.0324]	0.314
	T (Lafepe)	0.0273 [0.0253; 0.0309]	0.0282 [0.0257; 0.0326]	0.799
Vz/F (L)	R1 (Elea)	33.38 [27.35; 34.47]	43.09 [35.89; 48.08]	< 0.001***
	R2 (Liconsa)	30.54 [27.32; 36.35]	41.51 [38.10; 44.68]	< 0.001***
	T (Lafepe)	31.29 [25.57; 37.63]	39.33 [35.66; 44.07]	0.003***
Vz/F II (L/kg)	R1 (Elea)	0.48 [0.41; 0.54]	0.51 [0.49; 0.58]	0.062
	R2 (Liconsa)	0.47 [0.42; 0.53]	0.49 [0.48; 0.55]	0.067
	T (Lafepe)	0.47 [0.41; 0.50]	0.51 [0.43; 0.61]	0.129
Dose (mg/kg)#	R1 (Elea)	1.45 [1.40; 1.62]	1.21 [1.12; 1.47]	0.003***
	R2 (Liconsa)	1.48 [1.41; 1.60]	1.20 [1.12; 1.46]	0.002***
	T (Lafepe)	1.49 [1.41; 1.61]	1.20 [1.12; 1.47]	0.002***
Weight (kg)#	R1 (Elea)	69.05 [61.88; 71.25]	82.90 [67.85; 89.75]	0.003***
	R2 (Liconsa)	67.90 [62.38; 70.98]	83.40 [68.35; 89.35]	0.002***
	T (Lafepe)	67.35 [62.28; 71.00]	83.10 [68.20; 89.75]	0.003***

*Expressed as median [Q1; Q3]; **Mann-Whitney Rank Sum Test; ***p < 0.05. t_{max}: time to reach C_{max}; C_{max}: maximum concentration; C_{max}/D: maximum concentration corrected by dose according to weight; AUC_{84h}: area under the curve up to the last measurable concentration; AUC_{84h}/D: area under the curve up to the last measurable concentration corrected by dose according to weight; AUC_{∞}/D: area under the curve up to infinity; AUC_{∞}: area under the curve up to infinity corrected by dose according to weight; $\%AUC_{extrap}$: extrapolated percentage of AUC_{∞} in relation to AUC_{84h}; λ: apparent elimination constant; $t_{1/2}$: elimination half-life; CL/F: apparent total body clearance; CL/F II: apparent total body clearance as a function of weight; Vz/F: apparent volume of distribution; V_z/F II: apparent volume of distribution as a function of weight. #For dose normalisation weight was measured at each study period, which explains the variation in weight and dose (mg/kg) parameters between formulations.

plasma concentrations were higher in healthy women. The higher plasma exposure observed in women may also be partially explained by differences in body weight, as lower body weight may lead to relatively higher drug exposure when dosing is not adjusted by weight. The V_z/F were significantly higher in men for all formulations (between 26 and 36%). C_{max} were significantly greater in women for all formulations, but AUC were significantly greater in women for Elea and Lafepe formulations.

Interestingly, the influence of sex varied between formulations. Significant sex-related differences in t_{max} were observed for Elea and Liconsa formulations, but not for Lafepe, suggesting a slower absorption rate in women for these formulations. Women would have a slower gastric emptying time compared to men,[36] which agrees with what was observed for the Elea and Liconsa formulations. Additionally, only Elea and Lafepe formulations exhibited significant sex differences for AUC and clearance (CL/F; possibly influenced by AUC), whereas only Liconsa formulation showed significant differences in elimination variables λ and $t_{1/2}$. These observations could be attributed to aleatory variability of the data or to reflect a true sex-by-formulation effect.

The potential impact of the sex-by-formulation effects on bioequivalence studies has been discussed by some authors.[37,38,39] In agreement, regulatory agencies such as Anvisa and FDA recommend that bioequivalence studies included balanced male and female participation, unless the drug is intended solely for one sex, such as for hormonal contraceptives. In our study, no significant sex-by-formulation interactions were found, reinforcing the interchangeability of the three formulations across both sexes.

Regarding adverse events, 60% of those classified as probable, possible or conditional causality were observed in women. Similarly, other authors were reported a higher incidence of adverse events and treatment discontinuation among women in a cohort of 195 CD patients treated with BZN.[40] The reason for this sex difference is still unclear. Consistent with the higher plasma exposure observed in women in the present study, a recent clinical pharmacokinetic analysis in patients with chronic CD treated with the Lafepe formulation also reported higher BZN concentrations in women, largely explained by lower body weight.[17] The study further showed that higher plasma exposure, regardless of sex, was associated with an increased risk of skin reactions.[17] These findings suggest that weight-based dosing adjustments, rather than sex-specific dosing, may be more relevant for optimising treatment safety. Although differences in the frequency of adverse events were observed among formulations, these findings should be interpreted with caution due to the small sample size and the exploratory nature of the safety analysis. The study was not powered to detect statistically significant differences in safety outcomes between formulations.

Importantly, the present study focused on the 100 mg BZN formulation, which is commonly used in adult treatment. Further studies evaluating the pharmaceutical characteristics and bioequivalence of the 12.5 mg formulation used in paediatric populations would be valuable.

In conclusion - This was the first study to demonstrate bioequivalence among the three 100 mg BZN formulations currently available on the global market. This finding holds both clinical and economic relevance, as it supports a greater distribution of all three drugs across different markets, improving patient access. It also has scientific relevance, allowing for the comparison of results from different clinical trials, even when conducted with different formulations. Additionally, the study highlighted that sex may be a relevant factor contributing to BZN pharmacokinetic variability, which could have clinical implications, since women would be subjected to greater exposure to the drug in fixed-dose regimens.

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AUTHORS' CONTRIBUITION

GPES, LBF, DPP, ASS and EP designed the research; DPP and JFGMA were responsible for the analysis by mass spectrometry; GPES and RE were responsible for the pharmacokinetic analysis and statistical treatment of the data; LBF was responsible for the pharmaceutical equivalence analysis; GPES, LBF, FSNSM and LVPM planned the manuscript and wrote the introduction and discussion; DV and ASS revised the manuscript. All authors wrote the manuscript. There are no conflicts of interest among any of the researchers. This study was conducted as an independent initiative by the researchers, without any involvement from the pharmaceutical industry.

DATA AVAILABILITY

The data that support the findings of this study are not openly available due to reasons of sensitivity and are available from the corresponding author upon reasonable request. Data are located in controlled access data storage at Fundação Oswaldo Cruz.

REFERENCES

1. Sousa AS, Vermeij D, Ramos Jr AN, Luquetti AO. Chagas disease. Lancet. 2024; 403: 203-18. doi:10.1016/S0140-6736(23)01787-7.
2. Schmunis GA, Yadon ZE. Chagas disease: a Latin American health problem becoming a world health problem. Acta Trop. 2010; 115: 14-21. doi:10.1016/j.actatropica.2009.11.003.
3. PAHO/WHO - Pan American Health Organisation/World Health Organisation. Updated estimate of Chagas disease in the endemic countries of the Americas. 2018. Available from: https://www.paho.org/es/documentos/actualizacion-estimacion-enfermedad-chagas-paises-endemicos-americas-2018.
4. Porta EOJ, Kalesh K, Steel PG. Navigating drug repurposing for Chagas disease: advances, challenges, and opportunities. Front Pharmacol. 2023; 14: 1233253. doi:10.3389/fphar.2023.1233253.
5. Pérez-Molina JA, Crespillo-Andújar C, Bosch-Nicolau P, Molina I. Trypanocidal treatment of Chagas disease. Enferm Infecc Microbiol Clin (Engl Ed). 2021; 39: 458-70. doi:10.1016/j.eimce.2020.04.012.

6. PAHO/WHO - Pan American Health Organisation/World Health Organisation. Guidelines for the diagnosis and treatment of Chagas disease. Available from: https://www.paho.org/en/documents/guidelines-diagnosis-and-treatment-chagas-disease.

7. Coura JR, de Abreu LL, Willcox HP, Petana W. [Comparative controlled study on the use of benznidazole, nifurtimox and placebo, in the chronic form of Chagas' disease, in a field area with interrupted transmission. I. Preliminary evaluation]. Rev Soc Bras Med Trop. 1997; 30: 139-44. doi:10.1590/s0037-86821997000200009.

8. Kann S, Concha G, Frickmann H, Hagen RM, Warnke P, Molitor E, et al. Chagas disease: comparison of therapy with nifurtimox and benznidazole in indigenous communities in Colombia. J Clin Med. 2024; 13: 2565. doi:10.3390/jcm13092565.

9. Perin L, da Silva RM, Fonseca KS, Cardoso JMO, Mathias FAS, Reis LES, et al. Pharmacokinetics and tissue distribution of benznidazole after oral administration in mice. Antimicrob Agents Chemother. 2017; 61: e02410-16. doi:10.1128/AAC.02410-16.

10. Fragata-Filho AA, França FF, Fragata CS, Lourenço AM, Faccini CC, Costa CAJ. Evaluation of parasiticide treatment with benznidazol in the electrocardiographic, clinical, and serological evolution of Chagas disease. PLoS Negl Trop Dis. 2016; 10: e0004508. doi:10.1371/journal.pntd.0004508.

11. Morillo CA, Marin-Neto JA, Avezum A, Sosa-Estani S, Rassi A, Rosas F, et al. Randomized trial of benznidazole for chronic Chagas' cardiomyopathy. N Engl J Med. 2015; 373: 1295-306. doi:10.1056/NEJMoa1507574.

12. Raaflaub J, Ziegler WH. Single-dose pharmacokinetics of the trypanosomicide benznidazole in man. Arzneimittelforschung. 1979; 29: 1611-4.

13. Müller Kratz J, Bournissen FG, Forsyth CJ, Sosa-Estani S. Clinical and pharmacological profile of benznidazole for treatment of Chagas disease. Expert Rev Clin Pharmacol. 2018; 11: 943-57. doi :10.1080/17512433.2018.1509704.

14. Pinheiro E, Brum-Soares L, Reis R, Cubides J-C. Chagas disease: review of needs, neglect, and obstacles to treatment access in Latin America. Rev Soc Bras Med Trop. 2017; 50: 296-300. doi:https://doi.org/10.1590/0037-8682-0433-2016.

15. Silveira GPE. Pharmacokinetic analysis of benznidazole and disulfiram: an approach for Chagas disease. 2023. Available from: https://www.arca.fiocruz.br/handle/icict/65031.

16. Hernández Y, Marson ME, Fernández ML, Sued O, Frola C, Lloret SP, et al. Pharmacokinetics of two pharmaceutical presentations of benznidazole in adult *Trypanosoma cruzi* infected population. Mem Inst Oswaldo Cruz. 2025; 120: e240177. doi:10.1590/0074-02760240177.

17. Silveira GPE, Portela LF, Saavedra LB, Costa FP, Pinto DP, Carneiro FM, et al. Benznidazole pharmacokinetics in patients with chronic Chagas disease: association with demographic factors and adverse drug reactions. J Antimicrob Chemother. 2026; 81(1): dkaf416. doi: 10.1093/jac/dkaf416.

18. MS/ANVISA - Ministério da Saúde/Agência Nacional de Vigilância Sanitária. Resolução-RDC No. 31, de 11 de agosto de 2010. Dispõe sobre a realização dos Estudos de equiavalência farmacêutica e de perfil de dissolução comparativo. Brasília: Ministério da Saúde/Agência Nacional de Vigilância Sanitária; 2010. Available from: https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2010/res0031_11_08_2010.html.

19. MS/ANVISA - Ministério da Saúde/Agência Nacional de Vigilância Sanitária. Resolução da Diretoria Colegiada - RDC No. 56, de 8 de outubro de 2014. Dispõe sobre a Certificação de Boas Práticas para a realização de estudos de Biodisponibilidade/Bioequivalência de medicamentos e dá outras providências. Brasília: Ministério da Saúde/Agência Nacional de Vigilância Sanitária; 2014. Available from: https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2014/rdc0056_08_10_2014.pdf.

20. MS/ANVISA - Ministério da Saúde/Agência Nacional de Vigilância Sanitária. Resolução - RE 1.170, de 19 de abril de 2006. Brasília: Ministério da Saúde/Agência Nacional de Vigilância Sanitária; 2006. Available from: https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2006/res1170_19_04_2006.html.

21. US-DHHS/FDA/CDER - United States Department of Health and Human Services/Food and Drug Administration/Center for Drug Evaluation and Research. Guidance for industry: food-effect bioavailability and fed bioequivalence studies. 2002. Available from: https://www.fda.gov/files/drugs/published/Food-Effect-Bioavailability-and-Fed-Bioequivalence-Studies.pdf.

22. MS/ANVISA - Ministério da Saúde/Agência Nacional de Vigilância Sanitária. Resolução - RDC Nº 27, de 17 de maio de 2012. Dispõe sobre os requisitos mínimos para a validação de métodos bioanalíticos empregados em estudos com fins de registro e pós-registro de medicamentos. Brasília: Ministério da Saúde/Agência Nacional de Vigilância Sanitária; 2012. Available from: https://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2012/rdc0027_17_05_2012.html.

23. US-DHHS/FDA/CDER - United States Department of Health and Human Services/Food and Drug Administration/Center for Drug Evaluation and Research. Guidance for industry: bioequivalence guidance. 2006. Available from: https://www.fda.gov/media/70115/download.

24. EMA/CMPHU-US/DHHS/FDA - European Medicines Agency/Commitee for Medical Products for Human Use-United States/Department of Health and Human Services/Food and Drug Administration. Guideline on the investigation of bioequivalence. 2010. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-investigation-bioequivalence-rev1_en.pdf.

25. MS/CNS - Ministério da Saúde/Conselho Nacional de Saúde. Resolução No. 466, de 12 de dezembro de 2012. Available from: https://bvsms.saude.gov.br/bvs/saudelegis/cns/2013/res0466_12_12_2012.html.

26. Welling PG. Effects of food on drug absorption. Annu Rev Nutr. 1996; 16: 383-415. doi:10.1146/annurev.nu.16.070196.002123.

27. Kambayashi A, Sako K, Kondo H. Scintigraphic evaluation of the *in vivo* performance of dry-coated delayed-release tablets in humans. Eur J Pharm Biopharm. 2020; 152: 116-22. doi:10.1016/j.ejpb.2020.04.022.

28. Kambayashi A, Shirasaka Y. Food effects on gastrointestinal physiology and drug absorption. Drug Metab Pharmacokinet. 2023; 48: 100488. doi:10.1016/j.dmpk.2022.100488.

29. US-DHHS/FDA - United States Department of Health and Human Services/Food and Drug Administration. Highlights of prescribing information: benznidazole tablets. 2021. Available from: https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/209570s002lbl.pdf.

30. Wiens MO, Kanters S, Mills E, Lucano AAP, Gold S, Ayers D, et al. Systematic review and meta-analysis of the pharmacokinetics of benznidazole in the treatment of Chagas disease. Antimicrob Agents Chemother. 2016; 60: 7035-42. doi:10.1128/AAC.01567-16.

31. Molina I, Salvador F, Sánchez-Montalvá A, Artaza MA, Moreno R, Perin L, et al. Pharmacokinetics of benznidazole in healthy volunteers and implications in future clinical trials. Antimicrob Agents Chemother. 2017; 61: e01912-16. doi:10.1128/AAC.01912-16.

32. Raaflaub J, Ziegler WH. Single-dose pharmacokinetics of the Trypanosomicide benznidazole in man. Arzneimittelforschung. 1979; 29: 1611-4.

33. Jesus SM, Pinto L, Moreira FL, Nardotto GHB, Cristofoletti R, Perin L, et al. Pharmacokinetics of benznidazole in experimental chronic Chagas disease using the Swiss mouse-Berenice-78 *Trypanosoma cruzi* strain model. Antimicrob Agents Chemother. 2021; 65: e01383-20. doi:10.1128/AAC.01383-20.

34. Schwartz JB. The influence of sex on pharmacokinetics. Clin Pharmacokinet. 2003; 42: 107-21. doi:10.2165/00003088-200342020-00001.

35. Zucker I, Prendergast BJ. Sex differences in pharmacokinetics predict adverse drug reactions in women. Biol Sex Differ. 2020; 11: 32. doi:10.1186/s13293-020-00308-5.

36. Datz FL, Paul E, Moore C, Moore J. Gender-related differences in gastric emptying. J Nucl Med. 1987; 28(7): 1204-7.

37. Ibarra M, Vázquez M, Fagiolino P. Sex effect on average bioequivalence. Clin Ther. 2017; 39: 23-33. doi:10.1016/j.clinthera.2016.11.024.

38. González-Rojano E, Marcotegui J, Ochoa D, Román M, Álvarez C, Gordon J, et al. Investigation on the existence of sex-by-formulation interaction in bioequivalence trials. Clin Pharmacol Ther. 2019; 106: 1099-112. doi:10.1002/cpt.1539.

39. Chen ML, Lee SC, Ng MJ, Schuirmann DJ, Lesko LJ, Williams RL. Pharmacokinetic analysis of bioequivalence trials: implications for sex-related issues in clinical pharmacology and biopharmaceutics. Clin Pharmacol Ther. 2000; 68: 510-21. doi:10.1067/mcp.2000.111184.

40. da Silva GMS, Mediano MFF, Americano do Brasil PEA, Chambela MC, da Silva JA, de Sousa AS, et al. A clinical adverse drug reaction prediction model for patients with chagas disease treated with benznidazole. Antimicrob Agents Chemother. 2014; 58: 6371-7. doi:10.1128/AAC.02842-14.

OPEN PEER REVIEW

Memórias do IOC thanks the anonymous reviewers for their contribution to the peer review of this work.

FIRST REVIEW ROUND

EDITOR COMMENTS

Abstract: “The BZN Liconsa demonstrated faster in vitro dissolution”. This is not described in the Methods of the Abstract.

Abstract. “Plasma concentrations of BZN were higher in women”. For all formulations? How much higher? Include data.

Introduction “burdens,” Should be a full stop and not comma.

Introduction “over 75 million people are at risk” The last number is 100 million. (https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis))

Introduction “It acts by suppressing circulating forms of *T. cruzi*, potentially reducing or eliminating parasitemia”. Does it mean that BZN has no activity against the intracellular (amastigote) form?

Introduction “except in advanced chronic Chagas heart disease, where effectiveness tends to diminish with longer infection duration”. The BENEFIT trial demonstrated that BZN was effective to decrease parasitemia. BZN failed to show benefit on the studied clinical endpoints. It would be better to clarify this point.

Introduction “For over four decades, PK data were based on formulations that are no longer available, such as Rochagan® and Radanil®, previously produced by Hoffmann-La Roche”. There are some papers already published with PK data for LAFEPE and ELEA formulations (example: Silveira GPE. J Antimicrob Chemother. 2025 Nov 24: dkaf416) Please, discuss.

Methods: “T1/2” should read “t1/2”

Methods: Brazil is not signatory of the Declaration of Helsinki and the last version is 2024.

Methods: “The study protocol was published in the Brazilian Registry of Clinical Trials (ReBEC) with the following ID: 63382222.4.0000.80981”. Please include the link.

Results: “This difference can be attributed to the average body weight of male participants, which was approximately 19% higher than that of females. Consequently, the weight- adjusted dose (mg/kg) was about 18% lower in men”. As the beginning of the paragraph sates higher concentrations in women, it would be better to describe a lower weight in women and consequent larger weight-adjusted dose in women.

Results: There was a difference in Tmax between sexes that was not discussed.

Discussion

First paragraph. There are already some publications about PK using the current available BZ formulations.

Discussion. “The pharmacokinetic parameters observed in this trial were similar to those reported in previous studies [24,25], even those using formulations no longer on the market [26].” Please, rephrase this sentence to be clear which PK parameters are similar to studies that used the same formulation studied in the present paper, and the ones similar to papers that used other formulations.

Discussion. Please include a discussion comparing your results to a recent paper which included patients with CD. Silveira GPE. J Antimicrob Chemother. 2025 Nov 24: dkaf416.

Discussion “Although women exhibit higher BZN plasma concentrations following single-dose administration, the correlation between this higher exposure and adverse events remains uncertain [35]. Larger clinical studies in patients undergoing full treatment could help determine whether these sex-based differences impact therapeutic efficacy and safety at steady-state concentrations.” In fact, a clinical study was just published and should be included in this discussion. Silveira GPE. J Antimicrob Chemother. 2025 Nov 24: dkaf416

Author contribution “G.P.E.S., R.E., R.M.S. and M.A.V.S. designed the research.” Please clarify who RMS and MAVS stand for among the paper co-authors.

Data availability. Do you plan to include your data in the ARCA repository?

Figure 4. Use different colors for male and female.

Some Figures repeat information already presented by Tables. Please, suppress redundant information.

REVIEWERS’ COMMENTS

REVIEWER #1

1. Abstract

1.1. In the non-compartmental pharmacokinetic analysis, the following parameters are considered: AUC (area under the curve), Cmax, Tmax, elimination half-life ($t_{1/2}$), mean residence time (MRT), and clearance (CL). In this context, it is noted that the abstract does not report the latter parameter, which should be included to provide a more complete summary and to facilitate future research on the topic.

2. Introduction

2.1. Considering the sentence "However, BZN is more frequently used due to its simpler dosing regimen, shorter treatment duration, and potentially better tolerability regarding adverse events", it is noted that differences in dosing regimens between the drugs are mentioned. However, according to the current Brazilian guideline (Clinical Protocol and Therapeutic Guidelines for Chagas Disease, 2018 – Ministry of Health), the study context does not support any distinction in posology or treatment duration between these therapies. The authors are therefore requested to revise this sentence to improve clarity and ensure accurate interpretation by readers.

3. Material and Methods

3.1. Considering the critical role of validated analytical methods and calibrated equipment in bioequivalence studies, the authors are requested to provide calibration records for the equipment used, covering the period immediately preceding and during the study execution, to ensure metrological traceability, data reliability, and the validity of the reported results.

3.2. The authors are requested to provide the package inserts of the three drugs evaluated, as well as detailed information on the batches used in the study and the corresponding monetary value in US dollars at the time the study was conducted, in order to enhance methodological transparency and facilitate verification and interpretation of the data by future readers.

3.3. It is requested that, between lines 151 and 158, the manuscript specify whether any dietary restrictions were applied to the study participants during recruitment and study conduct, including the type of diet implemented and its methodological justification.

3.4. Clarification is requested regarding the rationale for administering the drug "30 minutes after a standardized high-fat meal – line 161", considering that, in clinical practice, individuals undergoing treatment for Trypanosoma cruzi infection are generally advised to avoid high-fat foods. In this context, it is essential that the authors explicitly present the methodological justification for this choice and discuss its compatibility with standard clinical management, in light of current care recommendations. Furthermore, this clarification is critical to support the interpretation of the causality of the observed adverse events, allowing distinction between effects attributable to the drug administration and those potentially related to the ingestion of the standardized high-fat meal provided 30 minutes prior to dosing.

3.5. Considering the sentence "Following each BZN administration, participants were clinically observed for 24 hours", clarification is requested regarding how this clinical monitoring was conducted, specifying whether the assessment was performed by a physician and/or nurse, as well as the location where the observation took place. Additionally, it is requested to indicate whether participant hospitalization was required during this period, and if so, to describe the location and the hospitalization protocol applied.

3.6. Regarding the sentence "… laboratory tests after the clinical phase …", it is requested that a detailed table of all laboratory tests performed be provided, including a comparison of participants' plasma parameters before and after drug administration, accompanied by the corresponding medians and interquartile ranges (IQR, 25–75%), stratified by sex.

3.7. Clarification is requested regarding the methodological choice to recruit participants exclusively from a single geographic location in Brazil — "A total of 42 healthy participants residing in Campinas, São Paulo, Brazil were enrolled in the bioequivalence study – line 264" — since such a strategy may introduce selection bias and consequently limit the generalizability of the findings to other populations and regional contexts.

3.8. The authors are requested to present the subgroup consisting of 22 female participants, including the medians and interquartile ranges (IQR, 25–75%) for the variables age and body mass index (BMI), following the description of the study participants (line 267).

3.9. It is requested to describe how the dose was adjusted to participants' body weight (mg/kg), including the criteria used for dose calculation and its application throughout the study.

3.10. It is requested that, between lines 316 and 318, the percentage values corresponding to each adverse drug event be presented, in the same manner as previously reported data, immediately following the numerical values.

3.11. It is requested that the manuscript specify whether the use of concomitant drugs was necessary to manage the adverse events observed in the study population, or, alternatively, if no additional pharmacological intervention was required.

3.12. It is noted that, between lines 329 and 359, no bibliographic references were provided to support some of the points discussed in the study. Although the literature on this topic is limited, a simple search in PubMed identified 63 articles related to the subject, which underscores the need to include relevant references to adequately substantiate the discussion presented.

3.13. It is requested that, on lines 364 and 365, where the expression "According to FDA data …" appears, the corresponding bibliographic reference be explicitly provided, in order to ensure proper traceability and support for the cited information.

REVIEWER #2

1. General Considerations

The revised manuscript is of significant scientific interest, presenting the results of a study comparing the bioavailability of three marketed 100 mg benznidazole formulations.

2. Abstract Adequacy

The abstract is concise and well-structured. However, in the Objective section (line 28), it should be explicitly stated that the bioequivalence assessment refers only to the 100 mg formulations, not to all available formulations. Similarly, in the Main Conclusion (line 42), clarify that the findings pertain specifically to the 100 mg benznidazole formulation.

3. Introduction

• Lines 59–62: It is strongly recommended to incorporate the latest epidemiological data published by PAHO/WHO (Update on Chagas Disease Estimates in Endemic Countries of the Americas, 2018. OPS/CDE/VT/25-0005 – Pan American Health Organization, 2025).

• Lines 67–68: When mentioning the higher frequency of benznidazole use compared to nifurtimox, emphasize that this is primarily due to its greater market availability. The PAHO/WHO 2018 guidelines recommend both benznidazole and nifurtimox interchangeably as first-line treatment options.

4. Results

• For better visualization, include a flowchart illustrating sample selection in the Bioequivalence Analysis section (line 263).

• Safety Analysis: Why was the increase in liver enzymes classified as a "conditional adverse event," given that this is a well-documented clinical adverse effect (lines 316–318)? Were baseline laboratory tests performed on volunteers? This should be clarified, as hepatic and gastrointestinal diseases are listed among exclusion criteria (Study Population, Blood Sampling, and Adverse Event Monitoring, line 153).

• Regarding the occurrence of adverse events differing among formulations (lines 308–322), an explanation is warranted. Although the sample size (N=11) is small, the differences (55%, 36%, and 9% for Liconsa, Lafepe, and Elea, respectively) merit discussion. Was any statistical analysis performed to assess these differences?

5. Discussion

• Line 341–342: The in vitro dissolution differences are not only between Lafepe and Liconsa but also between Lafepe–Elea and Liconsa.

• From line 424 onward, where sex-related variability is discussed, no mention is made of weight, despite its inclusion in the results.

• Among study limitations, note that only the 100 mg formulation was evaluated; the 12.5 mg formulation remains to be analyzed.

6. Conclusions

Line 451 should clearly state that the conclusions refer to the 100 mg benznidazole formulations.

7. Figures

There are two identical Figure 1s on pages 38 and 43. The title refers to dissolution profiles of the three formulations; it should specify that this is a pharmaceutical equivalence test.

Additionally, consider including a figure comparing the pharmaceutical equivalence test and the bioequivalence analysis in terms of dissolution profiles.

AUTHORS' RESPONSE TO THE REVIEWERS

Dear Editor and Reviewers,

We would like to sincerely thank you for the time and effort dedicated to reviewing our manuscript, as well as for your valuable comments and suggestions. We believe that your contributions have significantly improved the clarity, scientific rigor, and overall quality of the manuscript.

All comments were carefully considered, and the manuscript was revised accordingly. Detailed responses to each point raised by the reviewers are provided below, indicating how the manuscript was modified or, when applicable, explaining the rationale for maintaining the original approach.

We hope that the revised version adequately addresses all concerns raised and meets the standards of the journal.

Thank you once again for your thoughtful and insightful review.

Sincerely,

The Authors.

I. Editor comments

I.1. Abstract: "The BZN Liconsa demonstrated faster in vitro dissolution". This is not described in the Methods of the Abstract.

We thank the reviewer for this comment. A sentence indicating that in vitro dissolution testing was performed as part of the pharmaceutical equivalence assessment has been added to the Methods section of the Abstract.

I.2. Abstract. "Plasma concentrations of BZN were higher in women". For all formulations? How much higher? Include data.

We thank the reviewer for this important observation. The statement in the Abstract was revised to clarify that sex-related differences were observed for the Elea and Lafepe formulations, while no statistically significant difference was observed for Liconsa.

I.3. Introduction "burdens," Should be a full stop and not comma.

We thank the reviewer for identifying this typographical error. The punctuation was corrected.

I.4. Introduction "over 75 million people are at risk" The last number is 100 million. (https://www.who.int/news-room/fact-sheets/detail/chagas-disease-(american-trypanosomiasis)

We thank the reviewer for pointing this out. The estimate was updated according to the most recent PAHO/WHO documents.

I.5. Introduction "It acts by suppressing circulating forms of T. cruzi, potentially reducing or eliminating parasitemia". Does it mean that BZN has no activity against the intracellular (amastigote) form?

We thank the reviewer for this important comment. We agree that the original wording could be misinterpreted as suggesting that benznidazole acts only on circulating trypomastigotes. The U.S. Food and Drug Administration (FDA) label for benznidazole explicitly states that the drug is active against all three stages of T. cruzi (trypomastigotes, amastigotes, and epimastigotes) though sensitivity may vary by strain. However, the presence of dormant, non-replicative amastigotes can limit the effectiveness of therapy and contribute to persistence and relapse. The sentence in the Introduction has been revised to clarify this point and avoid potential misinterpretation.

I.6. Introduction "except in advanced chronic Chagas heart disease, where effectiveness tends to diminish with longer infection duration". The BENEFIT trial demonstrated that BZN was effective to decrease parasitemia. BZN failed to show benefit on the studied clinical endpoints. It would be better to clarify this point.

We agree with the reviewer and have clarified the interpretation of the BENEFIT trial.

I.7. Introduction "For over four decades, PK data were based on formulations that are no longer available, such as Rochagan® and Radanil®, previously produced by Hoffmann-La Roche". There are some papers already published with PK data for LAFEPE and ELEA formulations (example: Silveira GPE. J Antimicrob Chemother. 2025 Nov 24:dkaf416) Please, discuss.

We thank the reviewer for this comment. The manuscript was submitted before the publication of Silveira et al. (2025), which reports pharmacokinetic data without an explicit bioequivalence assessment, and has now been included. Historically, most PK data were generated using Roche formulations that are no longer available, while more recent studies evaluated Lafepe alone or compared Lafepe and Elea. To our knowledge, no published studies have directly compared Lafepe and Liconsa formulations. The present study provides the first comparative PK and bioequivalence assessment of three currently available formulations (Lafepe, Elea/Abarax, and Liconsa) in healthy volunteers. The manuscript has been revised accordingly.

I.8. Methods: "T1/2" should read "t1/2"

The notation was standardized throughout the manuscript to t1/2. "Tmax" was also changed to "tmax".

I.9. Methods: Brazil is not signatory of the Declaration of Helsinki and the last version is 2024.

We thank the reviewer for this clarification. The text was revised accordingly.

I.10. Methods: "The study protocol was published in the Brazilian Registry of Clinical Trials (ReBEC) with the following ID: 63382222.4.0000.80981". Please include the link.

The requested link was added.

I.11. Results: "This difference can be attributed to the average body weight of male participants, which was approximately 19% higher than that of females. Consequently, the weight- adjusted dose (mg/kg) was about 18% lower in men". As the beginning of the paragraph sates higher concentrations in women, it would be better to describe a lower weight in women and consequent larger weight-adjusted dose in women.

We agree that the paragraph could be clearer and revised the wording.

I.12. Results: There was a difference in Tmax between sexes that was not discussed.

We thank the reviewer for noting this point. The results section was expanded to address the observed sex-related differences in tmax.

I.13. Discussion. First paragraph. There are already some publications about PK using the current available BZ formulations.

We thank the reviewer for this comment. The manuscript was submitted before the publication of Silveira et al. (2025), which has now been included in the revised version. We would like to clarify that the original statement referred specifically to the fact that this is the first study comparing three currently available benznidazole formulations and combining a full pharmacokinetic and bioequivalence evaluation in healthy volunteers. The sentence has been slightly rephrased to improve clarity.

l.14. Discussion. "The pharmacokinetic parameters observed in this trial were similar to those reported in previous studies [24,25], even those using formulations no longer on the market [26]." Please, rephrase this sentence

to be clear which PK parameters are similar to studies that used the same formulation studied in the present paper, and the ones similar to papers that used other formulations.

There are no clinically meaningful differences between the pharmacokinetic parameters reported in previous studies of Roche benznidazole formulations and those reported in the recent studies by Silveira GPE et al. (The Journal of Antimicrobial Chemotherapy, 2025) and Hernández Y et al. (Memorias Do Instituto Oswaldo Cruz, 2024). All studies consistently report similar values for maximum plasma concentration (Cmax), time to maximum concentration (tmax), and area under the curve (AUC) following a single 100 mg oral dose in adults. We have added the most recent references to the text.

I.15. Discussion. Please include a discussion comparing your results to a recent paper which included patients with CD. Silveira GPE. J Antimicrob Chemother. 2025 Nov 24: dkaf416.

The manuscript was submitted before the publication of Silveira et al. (2025). These data have now been incorporated into the Discussion to compare the pharmacokinetic findings for the Lafepe formulation.

I.16. Discussion "Although women exhibit higher BZN plasma concentrations following single-dose administration, the correlation between this higher exposure and adverse events remains uncertain [35]. Larger clinical studies in patients undergoing full treatment could help determine whether these sex-based differences impact therapeutic efficacy and safety at steady-state concentrations." In fact, a clinical study was just published and should be included in this discussion. Silveira GPE. J Antimicrob Chemother. 2025 Nov 24: dkaf416

The manuscript was submitted before the publication of Silveira et al. (2025). The discussion was updated to include the recently published clinical study.

I.17. Author contribution "G.P.E.S., R.E., R.M.S. and M.A.V.S. designed the research." Please clarify who RMS and MAVS stand for among the paper co-authors.

The data were erroneously inserted and were removed.

I.18. Data availability. Do you plan to include your data in the ARCA repository?

We thank the reviewer for the suggestion. At this stage, due to controlled access to individual pharmacokinetic data, the dataset will remain available upon reasonable request to the corresponding author. However, the possibility of deposition in the ARCA repository will be evaluated.

I.19. Figure 4. Use different colors for male and female.

Figure 4 was revised to use different colors to distinguish better male and female participants.

I.20. Some Figures repeat information already presented by Tables. Please, suppress redundant information.

Figure 5 and Table 6 were removed. At the request of the other reviewers, a new table and a new figure were inserted.

II. Reviewer 1

II.1. Abstract: In the non-compartmental pharmacokinetic analysis, the following parameters are considered: AUC (area under the curve), Cmax, Tmax, elimination half-life (t1/2), mean residence time (MRT), and clearance (CL). In this context, it is noted that the abstract does not report the latter parameter, which should be included to provide a more complete summary and to facilitate future research on the topic.

We thank the reviewer for this suggestion. Although clearance (CL/F) was calculated as part of the non-compartmental analysis, it was not originally included in the abstract to maintain conciseness and focus on the primary bioequivalence parameters (Cmax, tmax and AUC). Given the 200-word limit for the abstract, only the most relevant results are presented here; however, all data are reported and discussed in the main text and tables 3 and 5.

II.2. Introduction: Considering the sentence "However, BZN is more frequently used due to its simpler dosing regimen, shorter treatment duration, and potentially better tolerability regarding adverse events", it is noted that differences in dosing regimens between the drugs are mentioned. However, according to the current Brazilian guideline (Clinical Protocol and Therapeutic Guidelines for Chagas Disease, 2018 – Ministry of Health), the study context does not support any distinction in posology or treatment duration between these therapies. The authors are therefore requested to revise this sentence to improve clarity and ensure accurate interpretation by readers.

We thank the reviewer for this important comment. We agree that, according to the Brazilian Clinical Protocol and Therapeutic Guidelines for Chagas Disease (Ministry of Health, 2018), both benznidazole and nifurtimox are recommended for a treatment duration of approximately 60 days. Therefore, the reference to a shorter treatment duration in the original sentence could lead to misinterpretation and has been removed.

The Brazilian guideline indicates that benznidazole may be administered up to three times daily, whereas nifurtimox is recommended in three divided doses per day. In addition, the prescribing information from the manufacturers provides further clarification regarding the usual dosing schedule: benznidazole (Lafepe and Elea formulations) is recommended to be administered in two daily doses, while nifurtimox (Bayer) is recommended to be administered three times daily.

Therefore, we will remove the mention of a shorter regimen, but we will retain the observation that the BZN dosage is simpler and better tolerated.

II.3. Material and Methods: Considering the critical role of validated analytical methods and calibrated equipment in bioequivalence studies, the authors are requested to provide calibration records for the equipment used, covering the period immediately preceding and during the study execution, to ensure metrological traceability, data reliability, and the validity of the reported results.

We thank the reviewer for this important comment. All analytical procedures used in this study were conducted in a certified analytical and bioanalytical laboratory by Brazilian Health Regulatory Agency (Anvisa), following validated methods and in accordance with Good Laboratory Practice (GLP) principles and applicable Brazilian regulatory requirements.

The analytical instruments used during the study were routinely calibrated and maintained according to the laboratory's internal quality assurance system, ensuring metrological traceability and data reliability. Calibration and maintenance records are maintained by the laboratory and are available for audit if required.

A clarifying statement has been added to the Methods section.

II.4. The authors are requested to provide the package inserts of the three drugs evaluated, as well as detailed information on the batches used in the study and the corresponding monetary value in US dollars at the time the study was conducted, in order to enhance methodological transparency and facilitate verification and interpretation of the data by future readers.

We thank the reviewer for this suggestion. The information regarding the batches used in the study is already included in the manuscript (Methods section). Specifically, the text states: "Prior to the bioequivalence study, pharmaceutical equivalence was assessed through in vitro tests, including physicochemical and dissolution evaluations. Three drug formulations were analyzed: Benznidazole Lafepe, manufactured by Lafepe (batch 21030012); Abarax, manufactured by Elea Phoenix S.A. (batch 3218); and Benznidazole Chemo, manufactured by Liconsa S.A. (batch LC60657)."

Package inserts are publicly available from the respective manufacturers.

Regarding the inclusion of the monetary value of the products, these elements are not typically reported in bioequivalence studies, as they do not affect the pharmacokinetic evaluation of the formulations. However, the formulations evaluated correspond to marketed products used in clinical practice, but market prices may vary substantially across countries and over time.

II.5. It is requested that, between lines 151 and 158, the manuscript specify whether any dietary restrictions were applied to the study participants during recruitment and study conduct, including the type of diet implemented and its methodological justification.

We thank the reviewer for this comment. Participants were instructed to follow standard dietary restrictions typically applied in pharmacokinetic studies, including overnight fasting prior to drug administration. A clarification has been added to the Methods section.

II.6. Clarification is requested regarding the rationale for administering the drug "30 minutes after a standardized high-fat meal – line 161", considering that, in clinical practice, individuals undergoing treatment for Trypanosoma cruzi infection are generally advised to avoid high-fat foods. In this context, it is essential that the authors explicitly present the methodological justification for this choice and discuss its compatibility with standard clinical management, in light of current care recommendations. Furthermore, this clarification is critical to support the interpretation of the causality of the observed adverse events, allowing distinction between effects attributable to the drug administration and those potentially related to the ingestion of the standardized high-fat meal provided 30 minutes prior to dosing.

We thank the reviewer for this important comment. The administration of the study drug following a standardized high-fat meal was performed according to regulatory recommendations for bioequivalence studies (Brazilian Health Regulatory Agency, Anvisa), which require evaluation under fed conditions to assess potential food effects on drug absorption. Standardized meal conditions are commonly used to reduce variability in drug absorption and to ensure consistency in pharmacokinetic comparisons between formulations. The standardized meal was administered to all participants under the same conditions, minimizing potential confounding effects on the comparison between formulations.

The use of a controlled meal in the clinical research setting does not aim to reproduce routine dietary recommendations for patients undergoing treatment for Chagas disease, but rather to ensure standardized experimental conditions during the pharmacokinetic evaluation.

A clarifying statement has been added to the Methods section.

II.7. Considering the sentence "Following each BZN administration, participants were clinically observed for 24 hours", clarification is requested regarding how this clinical monitoring was conducted, specifying whether the assessment was performed by a physician and/or nurse, as well as the location where the observation took place. Additionally, it is requested to indicate whether participant hospitalization was required during this period, and if so, to describe the location and the hospitalization protocol applied.

We thank the reviewer for this comment. The clinical monitoring procedures were already described in the manuscript (lines 171–175). Briefly, participants remained under clinical observation for 24 hours at the clinical research unit, with monitoring performed by trained medical staff, including vital signs assessment, protocol-specified laboratory evaluations, and monitoring for adverse reactions.

To improve clarity and facilitate identification of this information, the text has been slightly revised in the Methods section.

II.8. Regarding the sentence "… laboratory tests after the clinical phase …", it is requested that a detailed table of all laboratory tests performed be provided, including a comparison of participants' plasma parameters before and after drug administration, accompanied by the corresponding medians and interquartile ranges (IQR, 25–75%), stratified by sex.

We thank the reviewer for this suggestion. However, the manuscript already contains a substantial number of figures and tables, and the inclusion of these descriptive data would not add meaningful value to the analysis of the results. The study population consisted exclusively of healthy participants, with only individuals presenting normal test results or no clinically significant alterations being included. Laboratory safety assessments were conducted before and after the clinical phase, and all participants were discharged according to the same eligibility criteria (healthy participants). Consequently, the statistical analyses would be expected to yield values within normal laboratory ranges and without clinical relevance, even when stratified by sex.

A description of the laboratory tests performed was included in the section "Study Population, blood samples and adverse events monitoring".

II.9. Clarification is requested regarding the methodological choice to recruit participants exclusively from a single geographic location in Brazil — "A total of 42 healthy participants residing in Campinas, São Paulo, Brazil were enrolled in the bioequivalence study – line 264" — since such a strategy may introduce selection bias and consequently limit the generalizability of the findings to other populations and regional contexts.

Recruitment from a single clinical research center is common practice in bioequivalence studies to ensure standardized study procedures and controlled conditions. This design minimizes variability related to operational factors.

A clarification has been added to the Methods section.

II.10. The authors are requested to present the subgroup consisting of 22 female participants, including the medians and interquartile ranges (IQR, 25–75%) for the variables age and body mass index (BMI), following the description of the study participants (line 267).

We thank the reviewer for this suggestion. Indeed, the study included 22 female participants. As the study was not designed or powered to perform subgroup analyses by sex, a separate table was not considered necessary. However, to provide additional descriptive information, the median age and body weight for the female subgroup have now been included in the Results section. We preferred to include the variable body weight rather than BMI because the medication dose adjustment is based on weight. Women had a lower weight than men, and they were also shorter (median of 1.59m for women (IQR 1.56-1.66); median of 1.77m for men (IQR 1.72-1.81)), which makes the BMI less discriminating between the sexes.

II.11. It is requested to describe how the dose was adjusted to participants' body weight (mg/kg), including the criteria used for dose calculation and its application throughout the study No dose adjustment was applied for the bioequivalence analysis. The compared formulations were fixed-dose products (100-mg tablets), and each subject received a single dose of each product. For the analysis of sex-related differences, dose normalization by body weight (mg/kg) was performed by dividing the nominal administered dose (100 mg) by each participant's body weight. This normalized dose was then used to adjust the observed pharmacokinetic parameters (e.g., Cmax/normalized dose).

An explanation was included in the Methods.

II.12. It is requested that, between lines 316 and 318, the percentage values corresponding to each adverse drug event be presented, in the same manner as previously reported data, immediately following the numerical values.

We thank the reviewer for this suggestion. As recommended, the adverse drug events have been reformatted to present the values as number and percentage (n, %) immediately following the numerical counts. The corresponding section of the Results (lines 316–318) has been revised accordingly.

II.13. It is requested that the manuscript specify whether the use of concomitant drugs was necessary to manage the adverse events observed in the study population, or, alternatively, if no additional pharmacological intervention was required.

We thank the reviewer for this comment. In a small number of cases, participants received a single dose of dipyrone (1 g) for the management of headache during the clinical observation period. This information has been added to the manuscript to clarify the management of adverse events.

II.14. It is noted that, between lines 329 and 359, no bibliographic references were provided to support some of the points discussed in the study. Although the literature on this topic is limited, a simple search in PubMed identified 63 articles related to the subject, which underscores the need to include relevant references to adequately substantiate the discussion presented.

We thank the reviewer for highlighting this issue. Additional references have been included in the Discussion to support the statements presented in this section.

II.15. It is requested that, on lines 364 and 365, where the expression "According to FDA data …" appears, the corresponding bibliographic reference be explicitly provided, in order to ensure proper traceability and support for the cited information.

We thank the reviewer for this observation. The appropriate bibliographic reference supporting the statement based on FDA data has now been included in the manuscript.

III. Reviewer 2

III.1. Abstract Adequacy: The abstract is concise and well-structured. However, in the Objective section (line 28), it should be explicitly stated that the bioequivalence assessment refers only to the 100 mg formulations, not to all available formulations. Similarly, in the Main Conclusion (line 42), clarify that the findings pertain specifically to the 100 mg benznidazole formulation.

We thank the reviewer for this helpful suggestion. The Objective and the Main Conclusion in the Abstract have been revised to explicitly indicate that the findings refer to the 100 mg formulation.

III.2. Introduction. Lines 59–62: It is strongly recommended to incorporate the latest epidemiological data published by PAHO/WHO (Update on Chagas Disease Estimates in Endemic Countries of the Americas, 2018. OPS/CDE/VT/25-0005 – Pan American Health Organization, 2025).

We thank the reviewer for this suggestion. The most recent epidemiological estimates published by PAHO/WHO have now been incorporated.

III.3. Introduction. Lines 67–68: When mentioning the higher frequency of benznidazole use compared to nifurtimox, emphasize that this is primarily due to its greater market availability. The PAHO/WHO 2018 guidelines recommend both benznidazole and nifurtimox interchangeably as first-line treatment options.

We thank the reviewer for this important observation. We agree that both benznidazole and nifurtimox are recommended as first-line treatment options for Chagas disease in PAHO/WHO guidelines and may be used interchangeably in terms of therapeutic efficacy.

With regard to availability, benznidazole is currently produced by two manufacturers, whereas nifurtimox is provided by Bayer through a donation program coordinated by the PAHO Strategic Fund, which has expanded access to treatment, particularly for vulnerable populations in endemic regions.

In clinical practice, however, the more frequent use of benznidazole has also been associated with its generally simpler dosing regimen and a more favorable tolerability profile compared with nifurtimox, as reported in the literature.

To better reflect these aspects, the corresponding sentence in the Introduction has been revised.

III.4. Results. For better visualization, include a flowchart illustrating sample selection in the Bioequivalence Analysis section (line 263).

We thank the reviewer for this suggestion. A flowchart illustrating participant enrollment, inclusion in the pharmacokinetic analysis has been added to the manuscript to improve the visualization of the bioequivalence study population (new Figure 2).

III.5. Results. Safety Analysis: Why was the increase in liver enzymes classified as a "conditional adverse event," given that this is a well-documented clinical adverse effect (lines 316–318)? Were baseline laboratory tests performed on volunteers? This should be clarified, as hepatic and gastrointestinal diseases are listed among exclusion criteria (Study Population, Blood Sampling, and Adverse Event Monitoring, line 153).

We thank the reviewer for this important comment. Baseline laboratory tests were performed prior to drug administration to ensure eligibility of participants, including evaluation of hepatic parameters. The increase in liver enzymes observed during the study was classified as a conditional adverse event because the clinical relevance could not be definitively attributed to drug exposure based on a single isolated laboratory alteration without clinical symptoms.

To clarify this point, the manuscript has been revised to explicitly mention that baseline laboratory tests were performed and to better explain the classification of this event.

III.6. Results. Regarding the occurrence of adverse events differing among formulations (lines 308–322), an explanation is warranted. Although the sample size (N=11) is small, the differences (55%, 36%, and 9% for Liconsa, Lafepe, and Elea, respectively) merit discussion. Was any statistical analysis performed to assess these differences?

We thank the reviewer for this observation. The apparent differences in adverse event frequency among formulations should be interpreted with caution due to the small sample size (N=11 per sequence) and the exploratory nature of the safety analysis. No formal statistical comparison was performed for adverse events, as the study was not powered to detect safety differences between formulations. This point has now been clarified in the manuscript.

III.7. Discussion. Line 341–342: The in vitro dissolution differences are not only between Lafepe and Liconsa but also between Lafepe–Elea and Liconsa.

We thank the reviewer for this observation. In the in vitro dissolution analysis, the Lafepe and Elea formulations were considered equivalent, whereas the Liconsa formulation showed a different dissolution profile, with a faster benznidazole release. Therefore, the difference was observed between Liconsa and both Lafepe and Elea formulations.

We agree that the original wording could lead to ambiguity, and the text has been revised to clarify that Lafepe and Elea showed equivalent dissolution profiles, while Liconsa differed from both formulations.

III.8. Discussion. From line 424 onward, where sex-related variability is discussed, no mention is made of weight, despite its inclusion in the results.

We thank the reviewer for this suggestion. The Discussion has been revised to explicitly mention the potential influence of body weight on the observed sex-related variability in benznidazole exposure.

III.9. Discussion. Among study limitations, note that only the 100 mg formulation was evaluated; the 12.5 mg formulation remains to be analyzed.

We thank the reviewer for this suggestion. The objective of the present study was specifically to evaluate the pharmaceutical equivalence and bioequivalence of benznidazole 100 mg tablet formulations, which are the strengths most commonly used in adult treatment. Therefore, the evaluation of the 12.5 mg formulation was outside the scope of the present study.

However, we agree that the assessment of pediatric formulations represents an important area for future research. To acknowledge this point, a sentence highlighting this knowledge gap has been added to the Discussion section.

III.10. Conclusions. Line 451 should clearly state that the conclusions refer to the 100 mg benznidazole formulations.

We thank the reviewer for this suggestion. The Conclusions section has been revised.

III.11. Figures. There are two identical Figure 1s on pages 38 and 43. The title refers to dissolution profiles of the three formulations; it should specify that this is a pharmaceutical equivalence test.

We thank the reviewer for this observation. The duplicated figure has been corrected. In addition, the figure title has been revised to clarify that the dissolution profiles correspond to the pharmaceutical equivalence test.

III.12. Figures. Additionally, consider including a figure comparing the pharmaceutical equivalence test and the bioequivalence analysis in terms of dissolution profiles.

We appreciate the suggestion; however, the article already presents a substantial amount of data within the proposed objectives. A more detailed comparison of the in vivo data with those observed in vitro may be addressed in a future study.

SECOND REVIEW ROUND

REVIEWERS' COMMENTS

REVIEWER #1

I believe the authors have satisfactorily answered the reviewers' questions and have largely incorporated their suggestions. My position is to accept the manuscript for publication with the changes they made and proceed with the publication process.

REVIEWER #2

No comments.

DECISION: ACCEPT SUBMISSION | RECEIVED 25 OCTOBER 2025 | ACCEPTED 09 APRIL 2026