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PHARMACOLOGY

Pharmacokinetics and Tissue Distribution of Benznidazole after Oral Administration in Mice

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ABSTRACT Specific chemotherapy using benznidazole (BNZ) for Chagas disease during the chronic stage is controversial due to its limited efficacy and toxic effects. Although BNZ has been used to treat Chagas disease since the 1970s, few studies about the biodistribution of this drug exist. In this study, BNZ tissue biodistribution in a murine model and its pharmacokinetic profile in plasma were monitored. A bioanalytical high-performance liquid chromatography method with a UV detector (HPLC-UV) was developed and validated according to the European Medicines Agency for quantification of BNZ in organs and plasma samples prepared by liquid-liquid extraction using ethyl acetate. The developed method was linear in the BNZ concentration, which ranged from 0.1 to 100.0 μg/ml for plasma, spleen, brain, colon, heart, lung, and kidney and from 0.2 to 100.0 μg/ml for liver. Validation assays demonstrated good stability for BNZ under all conditions evaluated. Pharmacokinetic parameters confirmed rapid, but low, absorption of BNZ after oral administration. Biodistribution assays demonstrated different maximum concentrations in organs and similar times to maximum concentration and mean residence times, with means of 40 min and 2.5 h, respectively. Therefore, the biodistribution of BNZ is extensive, reaching organs such as the heart and colon, which are the most relevant organs affected by *Trypanosoma cruzi* infection, and also the spleen, brain, liver, lungs, and kidneys. Simultaneous analyses of tissues and plasma indicated high BNZ metabolism in the liver. Our results suggest that low bioavailability, instead of inadequate biodistribution, could be responsible for therapeutic failure during the chronic phase of Chagas disease.

KEYWORDS benznidazole, Chagas disease, *Trypanosoma cruzi*

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Benznidazole (BNZ) is a nitroheterocyclic compound used as specific chemotherapy for Chagas disease, which is an infection caused by the hemoflagellate protozoan *Trypanosoma cruzi*. Individuals infected by *T. cruzi* experience an initial acute phase with nonspecific symptoms, but about 5 to 10% of patients dies due to injury in the central nervous system, with edema and congestion (encephalomyelitis) or congestive heart

TABLE 1 Slope, *y*-intercept, and coefficient of determination of straight line equations for plasma and organs

Organ	Slope	*y* intercept	Coefficient of determination
Plasma	0.0790	−0.0023	0.9989
Spleen	0.0837	−0.0018	0.9981
Brain	0.0854	−0.0073	0.9962
Colon	0.1016	−0.0045	0.9991
Heart	0.0785	−0.0030	0.9988
Liver	0.0904	−0.0039	0.9978
Lung	0.1835	0.0022	0.9981
Kidney	0.0985	0.0035	0.9958

failure. Without treatment, the infection may remain throughout the life of the individual and progress to the chronic stage in an indeterminate form (also known as asymptomatic), during which the individual has positive serology without clinical signs. However, 20 to 30% of infected individuals develop the cardiac form (cardiomegaly, arrhythmias, or congestive heart failure) and 8 to 10% develop the digestive (reduced peristalsis, dysphagia, megacolon, and/or megaesophagus) or mixed form (1–3).

Despite serious side effects (4), the treatment of Chagas disease with BNZ is recognized as effective if administered during the acute phase, with 65 to 80% experiencing recovery (5). However, treatment during the chronic phase promotes recovery in only 37% of cases, with few protective effects during the clinical course of the disease (6, 7). However, some authors reported BNZ treatment as beneficial because the parasite is the major factor responsible for tissue lesions and the clinical course (8–10). Therefore, the usefulness of BNZ during the chronic phase is still undergoing investigation.

T. cruzi infection progresses when trypomastigote forms inside phagocyte cells differentiate into amastigote forms and replicate to invade other cells and tissues. Intracellular amastigotes can persist in a dormant state in the host body for decades by hiding in organs, thus causing progressive lesions and damage in the tissues of symptomatic patients (11). Whether BNZ inefficiency during the chronic phase could be due to inadequate biodistribution and low tissue penetration, especially in target organs affected by *T. cruzi*, was evaluated in this study.

BNZ tissue biodistribution has not been very well studied, and literature data are reported for only a few organs (12, 13). However, several methods have been described for BNZ quantification in animal and human plasma, urine, and milk (12–22). To evaluate BNZ biodistribution, a bioanalytical method was developed to determine its quantification in mouse tissues, including spleen, brain, heart, colon, liver, lung, and kidney. The method was also applied for simultaneous pharmacokinetic evaluation in plasma. The main goal of this study was to gain a detailed understanding of BNZ biodistribution in a murine model and its relation to target organs affected during the chronic phase of Chagas disease.

RESULTS

Validation. The bioanalytical method developed for quantification of BNZ in mice plasma and tissues by high-performance liquid chromatography (HPLC) was validated and proven to be selective without significant interference from biological matrices or any residual effects from previously analyzed samples (data not shown). The method was linear for all tissues analyzed, with a correlation coefficient (r) of >0.99 (Table 1). The precision and accuracy of intrarun and interrun coefficients of variation and relative standard error, respectively, were within acceptable limits determined by the European Medicines Agency (EMA) (23), thus confirming the reproducibility and reliability of the method (Table 2). The stability of BNZ after different handling conditions required by the method was also confirmed (Table 3). The standard solutions prepared in acetonitrile showed good stability, with mean relative standard errors of 0.92% and −3.98% for

TABLE 2 Precision and accuracy of intrarun and interrun for samples processed with the lower limit of quantitation and low, middle, and high quality control levels[a]

Sample	Nominal concn[b]	% obtained from nominal concn	Precision (% CV)		Accuracy (% RSE)	
			Intrarun	Interrun	Intrarun	Interrun
Plasma	0.10	105.12	2.92	3.98	5.12	1.54
	0.25	106.63	3.57	4.72	6.63	9.40
	50.00	92.08	6.36	4.22	−7.92	−4.90
	75.00	102.03	2.00	8.70	2.03	−0.03
Spleen	0.10	116.07	12.23	8.63	16.07	6.73
	0.25	95.30	7.79	7.36	−4.70	−6.08
	50.00	98.08	3.12	7.13	−1.92	−0.27
	75.00	101.05	2.81	6.17	1.05	0.47
Brain	0.10	112.72	8.30	12.14	12.72	15.76
	0.25	90.29	10.44	7.94	−9.71	−3.02
	50.0	88.01	9.35	8.37	−11.99	−8.72
	75.0	99.59	4.88	4.11	−0.41	1.90
Colon	0.10	115.59	6.22	9.30	15.59	13.47
	0.25	105.17	4.08	7.43	5.17	5.31
	50.00	93.99	4.17	5.97	−6.01	−0.71
	75.00	92.55	4.10	4.20	−7.45	−2.69
Heart	0.10	108.56	6.26	11.86	8.56	8.68
	0.25	105.57	4.86	6.05	5.57	3.16
	50.00	100.17	3.32	5.54	0.17	−4.08
	75.00	101.95	5.33	6.07	1.95	−4.03
Liver	0.20	97.73	3.47	6.80	−2.27	−0.87
	0.50	98.56	4.80	7.19	−1.44	−0.93
	50.00	91.87	2.46	12.84	−8.13	−1.75
	75.00	100.32	1.03	5.94	0.32	1.94
Lung	0.10	102.32	12.51	9.11	2.32	2.75
	0.25	87.93	10.01	10.29	−12.07	−3.54
	50.0	103.51	5.25	7.31	3.51	1.21
	75.0	102.21	8.07	7.76	2.21	−0.96
Kidney	0.10	104.18	5.25	12.87	4.18	−11.89
	0.25	105.42	5.34	4.10	5.42	−7.38
	50.0	99.01	5.33	6.15	−0.99	−0.68
	75.0	105.35	1.11	3.00	5.35	3.06

[a]Precision and accuracy were determined using an analytical method. CV, coefficient of variation; RSE, relative standard error.
[b]Nominal concentrations are in micrograms per milliliter (plasma) or micrograms per gram (tissue).

BNZ and the internal standard (IS), respectively. All results obtained with validation assays were in accordance with the specifications of the EMA (23).

Pharmacokinetics and biodistribution. BNZ systemic exposure profiles were obtained by plasma and tissue concentration-versus-time curves (Table 4). Data were fitted using the monocompartment model to calculate pharmacokinetic parameters (Table 5).

The time to reach maximum concentration (T_{max}) in plasma was 0.83 h, and the maximum concentration (C_{max}) in plasma was 41.61 μg/ml. In this study, the elimination half-life ($t_{1/2b}$) of BNZ was 2.03 h, and mean residence time (MRT) was 3.86 h. The volume of distribution (V) and clearance (CL), both as a function of BNZ bioavailability (F), were 38.81 ml and 13.29 ml/h, respectively.

Biodistribution was evaluated by calculating the pharmacokinetic parameters in tissues. The C_{max} in tissues ranged from 1.76 μg/g in the liver to 34.58 μg/g in the lung. The T_{max} ranged from 0.49 h in the kidney to 0.75 h in the liver and brain. MRT was shorter in the spleen (1.79 h) and longer in the liver (3.41 h). All other biodistribution parameters are listed in Table 6.

TABLE 3 Short-term, on-instrument autosampler, freeze-thaw, and long-term stabilities of samples processed with low and high quality control levels[a]

Sample	NC[b]	Short-term stability			On-instrument autosampler stability			Freeze-thaw stability			Long-term stability		
		% obtained from NC	Precision (% CV)	Accuracy (% RSE)	% obtained from NC	Precision (% CV)	Accuracy (% RSE)	% obtained from NC	Precision (% CV)	Accuracy (% RSE)	% obtained from NC	Precision (% CV)	Accuracy (% RSE)
Plasma	0.25	107.39	1.91	7.39	105.95	7.70	5.95	108.59	6.51	8.59	95.00	9.07	−5.00
	75.00	92.17	7.15	−7.83	94.47	2.19	−5.53	94.96	3.54	−5.04	97.23	9.95	−2.77
Spleen	0.25	101.38	7.24	1.38	101.19	5.36	1.19	95.63	4.81	−4.37	97.40	9.85	−2.60
	75.00	100.31	12.22	0.31	98.58	2.10	−1.42	104.61	7.69	4.61	103.44	6.35	3.44
Brain	0.25	90.93	6.59	−9.07	97.35	5.04	−2.65	98.27	1.55	−1.73	93.62	4.77	−6.38
	75.0	91.42	8.36	−8.58	95.11	6.14	−4.89	98.06	3.74	−1.94	91.20	10.65	−8.80
Colon	0.25	101.08	5.48	1.08	104.78	3.44	4.78	92.91	4.72	−7.09	101.89	11.65	1.89
	75.00	93.61	4.24	−6.39	92.01	4.00	−7.99	96.49	3.38	−3.51	110.99	7.55	10.99
Heart	0.25	90.68	10.67	−9.32	112.38	1.56	12.38	88.14	5.50	−11.86	84.19	5.64	−14.40
	75.00	95.93	4.04	−4.07	89.82	2.22	−10.18	105.77	4.35	5.77	104.43	1.58	4.43
Liver	0.50	108.67	6.74	8.67	100.32	12.07	0.32	96.85	10.92	−3.15	96.29	10.57	−3.71
	75.00	102.78	5.31	2.78	95.65	11.68	−4.35	95.55	9.19	−4.45	99.79	4.08	−0.21
Lung	0.25	89.90	8.02	−10.10	101.77	11.11	1.77	94.78	7.68	−5.22	92.44	4.51	−7.56
	75.0	99.06	7.15	−0.94	92.58	10.20	−7.42	92.03	10.29	−7.97	100.69	4.57	0.69
Kidney	0.25	89.94	3.43	−10.06	88.11	6.80	−11.89	91.64	4.18	−8.36	104.15	4.96	4.15
	75.0	102.95	2.19	2.95	103.94	0.99	3.94	98.41	5.07	−1.59	107.94	13.92	7.94

[a]Precision and accuracy were determined using an analytical method. CV, coefficient of variation; RSE, relative standard error.
[b]Nominal concentrations (NC) are in micrograms per milliliter (plasma) or micrograms per gram (tissue).

DISCUSSION

The bioanalytical method proposed in this work to quantify BNZ in plasma and tissues of mice is in agreement with the specifications of the EMA (23). By following these validation guidelines, the stability of BNZ under different conditions required by the method was confirmed. Moreover, the method was sensitive, precise, accurate, and linear for BNZ concentrations ranging from 0.10 or 0.20 to 100.0 μg/ml, depending on the organ.

Testing of pharmacokinetics and biodistribution of the BNZ was performed using a dose of 100 mg/kg. Currently, Chagas disease patients are treated with a BNZ dosage of 5 to 10 mg/kg/day, not to exceed 300 mg/day (24). On the other hand, the reference dose for treatment of mice in experimental infection with *T. cruzi* is 100 mg/kg/day (25). The murine model needs a higher dose of a given compound to produce the same effects as in humans (26). The therapeutic scheme used corresponds to the maximum effective reported for mice infected with different strains (27, 28).

In this study, the time to reach maximum plasma concentration was short, with an average of 0.83 h. In Wistar rats treated orally, Leonardi et al. and Davanço et al. found

TABLE 4 BNZ concentrations in plasma and organs of mice after a single oral dose of 100 mg/kg ($n = 5$)

Time (h)	BNZ concn (mean ± SD)[a]							
	Plasma	Spleen	Brain	Colon	Heart	Liver	Lung	Kidney
0.16	8.83 ± 0.46	4.48 ± 0.32	0.80 ± 0.17	8.40 ± 6.60	2.43 ± 1.99	0.5 ± 0.21	8.19 ± 1.35	1.60 ± 0.58
0.33	25.60 ± 1.37	19.69 ± 2.34	8.68 ± 1.14	9.44 ± 3.34	15.93 ± 1.75	0.42 ± 0.19	25.13 ± 0.97	6.12 ± 0.17
0.50	39.00 ± 1.50	23.67 ± 4.90	9.50 ± 2.46	17.61 ± 2.17	20.13 ± 3.41	0.55 ± 0.25	25.73 ± 6.50	7.26 ± 0.13
0.75	37.59 ± 2.97	26.16 ± 2.73	12.04 ± 1.55	15.12 ± 1.86	17.99 ± 0.38	1.76 ± 0.19	33.20 ± 4.05	3.84 ± 1.59
1	41.97 ± 4.47	19.00 ± 2.38	10.13 ± 0.69	13.26 ± 3.53	16.99 ± 4.88	0.68 ± 0.08	26.97 ± 3.46	3.26 ± 0.78
2	38.02 ± 1.05	5.61 ± 0.69	8.18 ± 1.55	12.65 ± 1.73	13.31 ± 1.27	0.35 ± 0.03	26.74 ± 1.49	2.61 ± 0.25
3	25.99 ± 1.62	4.47 ± 1.55	7.00 ± 3.11	8.01 ± 2.80	9.58 ± 2.20	0.24 ± 0.02	19.31 ± 2.98	2.17 ± 0.25
4	21.54 ± 2.82	2.25 ± 0.51	0.65 ± 0.06	3.20 ± 0.83	3.45 ± 2.12	0.23 ± 0.03	8.73 ± 2.02	0.75 ± 0.07
5	13.05 ± 0.83	1.17 ± 0.06	ND	2.45 ± 2.28	ND	0.25 ± 0.01	5.13 ± 1.23	0.29 ± 0.06
6	13.06 ± 2.32	1.44 ± 0.06	ND	0.35 ± 0.00	ND	0.27 ± 0.00	4.43 ± 1.07	0.31 ± 0.00

[a]BNZ concentrations are in micrograms per milliliter per hour (plasma) or microgram per gram per hour (tissue). ND, not detected.

TABLE 5 Pharmacokinetic parameters of BNZ in mouse plasma after single oral dose of 100 mg/kg ($n = 5$)

Pharmacokinetic parameter[a]	Value (mean ± SD)
k_{el} (h^{-1})	0.34 ± 0.03
$t_{1/2b}$ (h)	2.03 ± 0.16
k_a (h^{-1})	2.23 ± 0.41
$t_{1/2a}$ (h)	0.29 ± 0.03
AUC_{0-6} (μg/ml · h)	154.53 ± 1.57
$AUC_{0-\infty}$ (μg/ml · h)	195.71 ± 6.04
MRT (h)	3.86 ± 0.23
T_{max} (h)	0.83 ± 0.29
C_{max} (μg/ml)	41.61 ± 2.99
V/*F* (ml)	38.81 ± 2.02
CL/*F* (ml/h)	13.29 ± 0.41

[a]k_{el}, elimination constant; $t_{1/2b}$, elimination half-life; k_a, absorption constant; $t_{1/2a}$, absorption half-life; AUC_{0-6}, area under the concentration-time curve from 0 to 6 h; $AUC_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; MRT, mean residence time; T_{max}, time to achieve C_{max}; C_{max}, maximum concentration; *V*, volume of distribution; CL, clearance; *F*, bioavailability.

BNZ T_{max}s of 2.0 and 1.1 h, respectively (17, 18). Moreira da Silva et al. observed BNZ T_{max} of 2.2 h in Swiss mice after oral treatment (16). Workman et al. found T_{max}s of 15, 30, or 60 min, depending on the dose, in BALB/c mice following intraperitoneal treatment and T_{max}s of 1 to 5 h for dogs treated orally (12).

The maximum plasma drug concentration observed in the present study was 41.61 μg/ml, on average, representing fast, but low, absorption of BNZ. It is well known that the major limiting step for BNZ absorption is its low water solubility (0.4 mg/ml) (17). Workman et al. reported a similar C_{max}, 36 μg/ml, in mice treated with 78 mg/kg of BNZ (12). In this study, the elimination half-life ($t_{1/2b}$) of BNZ was 2.03 h, which is similar to the values reported by Davanço et al., Moreira da Silva et al., and Workman et al., which were 2.7, 1.6, and 1.6 h, respectively (12, 16, 18). These short half-lives indicate a high metabolism, which is responsible for low oral bioavailability. However, Morilla et al. observed a BNZ $t_{1/2b}$ of 3.5 h after intravenous administration (14). This result was not expected, because the $t_{1/2b}$ of an orally administered drug should be longer than that of an intravenously administered one due to the absorption phase (18).

The volume of distribution (*V*; 38.81 ml) and clearance (CL; 13.29 ml/h), both as a function of BNZ bioavailability (*F*), and MRT (3.86 h) were not similar to any value reported by other authors, probably due to different doses of BNZ administered and/or different pharmacokinetic models used for data analysis. In addition, animal age is an important factor in the variation. According to Bulffer et al., young rats displayed higher BNZ levels and longer BNZ persistence times in plasma than did adults (15). Differences in BNZ pharmacokinetic parameters and biodistribution may be related also to the formulation used (13, 14, 17).

In biodistribution assays, the BNZ C_{max}s varied among different tissues. This is because the drug has different affinities for various tissue structures (29). However, using the pharmacokinetic approach, the organs can be grouped as a single compartment when a drug is rapidly and reversibly distributed among them (30). This is also the

TABLE 6 BNZ pharmacokinetic parameters in plasma and tissue biodistribution in mice after single oral dose of 100 mg/kg ($n = 5$)[a]

Sample	AUC_{0-6} (μg/ml · h or μg/g · h)	*R* (%)	$AUC_{0-\infty}$ (μg/g · h)	MRT (h)	T_{max} (h)	C_{max} (μg/ml or μg/g)
Plasma	154.53 ± 1.57	100	195.71 ± 6.04	3.86 ± 0.23	0.83 ± 0.29	41.61 ± 2.99
Spleen	42.39 ± 3.41	27.4	44.11 ± 3.54	1.79 ± 0.11	0.58 ± 0.14	27.73 ± 2.14
Brain	28.76 ± 4.32	18.6	29.45 ± 4.23	2.01 ± 0.24	0.75 ± 0.25	12.39 ± 1.70
Colon	45.64 ± 6.73	29.5	46.09 ± 6.71	2.03 ± 0.22	0.67 ± 0.29	18.11 ± 1.32
Heart	49.42 ± 7.07	31.9	57.78 ± 10.24	2.42 ± 0.58	0.67 ± 0.29	21.68 ± 2.70
Liver	2.24 ± 0.16	1.4	2.86 ± 0.04	3.41 ± 0.17	0.75 ± 0.00	1.76 ± 0.19
Lung	96.06 ± 3.34	62.2	106.14 ± 5.34	2.74 ± 0.17	0.63 ± 0.13	34.58 ± 2.87
Kidney	11.75 ± 0.75	7.6	12.20 ± 0.74	2.00 ± 0.03	0.49 ± 0.02	6.98 ± 0.51

[a]Values are means ± SDs. *R*, $AUC_{0-6,\ tissue}$ /$AUC_{0-6,\ plasma}$.

case for BNZ, as confirmed by its plasma concentration-versus-time profile, which was best fitted by the monocompartmental pharmacokinetic model.

BNZ was detected in the brain and heart of mice up to 4 h after treatment and in others tissues during the analysis time. The ratio for the BNZ brain/plasma area under the curve from 0 to 6 h (AUC_{0-6}) was 19% (Table 6). Workman et al., using HPLC-UV as an analytical technique, showed brain/plasma BNZ mean ratios of 68.3% and 42% in mice and dogs treated intraperitoneally and orally, respectively (12).

The possible differences in the permeation of BNZ through blood-brain barrier in animals and humans are not clear, but as shown above, differences between species may occur. These data suggest that BNZ can cross the blood-brain barrier and exert its action in cases of central nervous system parasitism. However, other studies have indicated that BNZ has toxic effects in the central nervous system. Dogs orally treated with BNZ presented encephalopathy with multifocal characteristics and clinical, pathological, and neurological disorders that were dose dependent and time dependent (31, 32). The results of these works reinforce the issue of BNZ toxicity.

This study did not extend the analysis of the central nervous system to the bone marrow due to the impracticality of collecting enough material for the development and validation of the method for quantification of BNZ in this matrix. In addition, the involvement of this organ in *T. cruzi* infection is rarely observed (33).

In the lung, a BNZ lung/plasma AUC_{0-6} ratio of 62% and C_{max} of 34.58 μg/g were observed. These were the highest values among the tissues, probably due to the high vascularization of this organ.

The spleen AUC_{0-6} of mice in this experiment was equivalent to 27% of the plasma AUC_{0-6}, which is explained by high vascularization for blood filtration (34). Regarding *T. cruzi* infection, BNZ biodistribution in the spleen may have important clinical implications in the acute phase of Chagas disease, which is often responsible for splenomegaly. Olivieri et al. showed that on the ninth day after infection, infected mice treated with BNZ exhibited a significant reduction in spleen weight compared to that of untreated animals (35). This finding shows that the previous treatment results in lower parasitemia and, consequently, fewer changes in the immune system of the host (35).

The *in vitro* effect of BNZ is observed in both the amastigote and trypomastigote forms of *T. cruzi*, and it was already demonstrated the BNZ action in cells infected by the parasite (36). However, it is unclear what amount of BNZ can reach intracellular parasite forms, prevalent in chronic infection.

Regarding the liver, Workman et al. reported that an average of 41.7% and 70.6% of plasma concentrations were observed in the liver of BALB/c and C3H/He mice, respectively, after intraperitoneal administration of 78 mg/kg of BNZ (12). Morilla et al. reported that after intravenous administration of 0.2 mg/kg of BNZ in Wistar rats, 9.1% and 0.63% of the administered dose were observed in liver and kidney, respectively (13). In the present study, very low BNZ concentrations were observed in the livers and kidneys of Swiss mice after oral administration. The BNZ liver/plasma AUC_{0-6} ratio was only 1% (Table 6). When BNZ is orally administered, it is metabolized in the liver by the cytochrome P450 enzyme system, generating 2-amino-imidazole by reduction of the nitro group and 2-hydroxy-imidazole by hydrolytic replacement of the nitro group, which are excreted in the urine (12, 37). This process reduces the systemic bioavailability of BNZ. Our data show that the liver had a lower BNZ C_{max} (1.76 μg/ml) and the highest BNZ MRT (3.41 h), probably due to first-pass metabolism during the absorption phase. This is in accordance with the short half-life (2 h) observed in plasma. However, to understand how metabolism affects BNZ concentrations in the liver and, consequently, in the whole body, it is necessary to perform metabolism studies using a method for simultaneous quantification of BNZ and its metabolites. The *in vivo* effect of BNZ on biotransformation enzymes and transporters was already evaluated in mice and suggests the possibility of a progressive decrease in BNZ absorption and/or increase in BNZ metabolism/elimination after therapeutic administration. The overexpression of transporters can result in faster biliary excretion of drugs, increased secre-

tion of substances that are present in blood, and/or a reduced absorption of drugs that are orally administered (26).

All biodistribution parameters for the heart and colon were very similar, with the greatest values for heart/plasma and colon/plasma AUC_{0-6} ratios being 32% and 30%, respectively. These data are interesting because the heart and colon are the most relevant organs involved in *T. cruzi* infection. The acute phase of Chagas disease becomes fatal in cases of congestive heart failure; cardiac and digestive forms of the disease in the symptomatic chronic phase are also fatal (1, 3). Nevertheless, although large amounts of BNZ can reach the heart and colon, as represented by the AUC_{0-6} ratios in relation to plasma, the maximum concentration in plasma may be insufficient for complete parasite elimination in organs during the chronic phase of *T. cruzi* infection. Recently, the improvement of Chagas disease chemotherapy has been experimentally evaluated with new formulations of BNZ, such as extended-release tablets, solid dispersions, microparticles, and nanoparticles, to increase the maintenance of the drug concentration *in vivo* (38–40).

In summary, BNZ in *T. cruzi* infection has limited efficacy, especially during the chronic phase of the disease, when the parasites are mostly confined to deep tissues in which replication occurs (41, 42). According to the present study, BNZ biodistribution occurs broadly, reaching the heart and colon, which are the most relevant organs for *T. cruzi* infection, and also the spleen, brain, liver, lungs, and kidneys. Therefore, the limited efficacy of BNZ may be due not to limited tissue penetration but rather to low absorption during first-pass metabolism in the liver; both of these processes occur before tissue biodistribution. These findings need to be taken into account in further research of new drug delivery systems to improve BNZ pharmacokinetics.

MATERIALS AND METHODS

Chemicals and reagents. Analytical standard BNZ (*N*-benzyl-2-nitro-1H-imidazole-1-acetamide; 97% purity; lot MKBL3727V) and internal standard (IS) omeprazole {5-methoxy-2[[(4-methoxy-3,5-dimethyl-2-pyridinyl) methyl]sulfinyl]-1H-benzimidazole; 99% purity; lot BCBL3570V} were purchased from Sigma-Aldrich (St. Louis, MO). High-performance liquid chromatography (HPLC)-grade acetonitrile was obtained from J.T.Baker-Hexis (Deventer, the Netherlands) and analytical-grade ethyl acetate was obtained from TEDIA (Rio de Janeiro, Brazil). Ultrapure water was produced in the laboratory by distillation and purification with a Milli-Q Simplicity (Millipore) system. BNZ tablets (lot 13030214) were produced by Laboratório Farmacêutico do Estado de Pernambuco (LAFEPE; Pharmaceutical Laboratory of Pernambuco State, Recife, Brazil).

Preparation of standard solutions. Stock solutions of BNZ and IS analytical standards were prepared in acetonitrile at concentrations of 2 and 1 mg/ml, respectively. BNZ and IS working solutions were prepared from dilutions of the stock solution in acetonitrile and stored in polypropylene tubes at −80°C.

The calibration standards and quality control samples for the analytical method were prepared in 90 μl of blank plasma or blank macerated tissues with 5 μl of IS and 5 μl of BNZ working solutions. The final concentrations were 50 μg/ml for IS and 0.10, 0.20, 0.25, 0.50, 1.0, 10.0, 25.0, 50.0, 75.0, and 100.0 μg/ml for BNZ.

The quantification method for BNZ in mouse plasma and organs was validated by following the guidelines of the European Medicines Agency (EMA)–Science Medicines Health (23).

Chromatography conditions. Samples were analyzed by HPLC (E2695; Waters, Milford, MA) with a UV detector (Waters 2489) using an analytical C_{18} column (Gemini-NX; Phenomenex, Torrance, CA; 150 mm by 4.6 mm by 5 μm) and a C_{18} column guard (model AJ0-4287; Phenomenex; 4 mm by 3 mm by 5 μm) maintained at 40°C. The mobile phase was composed of an isocratic mixture of acetonitrile and ultrapure water (30:70, vol/vol) with a flow rate of 1.0 ml/min. Detection was monitored at 324 nm, and the injection volume was 20 μl.

Animals. Female Swiss mice (age, 30 days; weight, 25 to 30 g) were kept in a controlled room with regular alternating cycles of light at a temperature of 23 ± 2°C, given water *ad libitum*, and fasted 12 h prior to treatment. All procedures were performed according to the guidelines established by the National Council for Animal Experimentation Control (CONCEA) and approved by the Ethical Committee on Animal Experimentation of the Universidade Federal de Ouro Preto, Minas Gerais, Brazil (protocol 2013/59).

Treatment schedule, sample collection, and preparation. One tablet of BNZ (LAFEPE) was powdered and suspended in 10 ml of 0.5% carboxymethyl cellulose solution. Animals received a single dose of 100 mg/kg of BNZ by gavage and were euthanized 0.16, 0.33, 0.50, 0.75, 1, 2, 3, 4, 5, and 6 h after treatment. The heart, liver, spleen, lungs, kidneys, colon, and brain were collected from five animals at a time. Approximately 400 μl of blood was also collected from the orbital sinuses in polypropylene tubes with EDTA as an anticoagulant. Blood and organs of untreated animals were used as blank matrices for validation assays.

For plasma separation, blood samples were centrifuged at 8,609 × *g* and 18°C for 10 min. Organs *in toto* were washed in phosphate-buffered saline (PBS; pH 7.4), dried on filter paper, weighed, and stored at −80°C. Subsequently, the tissues were thawed, macerated (TissueLyser II; Qiagen, Hilden, Germany), and frozen again for future BNZ extraction procedures and quantification by HPLC. Preparation of the experimental samples was similar to that of the calibration standards and quality control samples; they were added to 5 μl of IS and 5 μl of acetonitrile instead of BNZ working solutions.

Samples were prepared by liquid-liquid extraction with the addition of 500 μl of ethyl acetate, vortexing for 10 min, and centrifugation for 10 min at 6,973 × *g*. Thereafter, 400 μl of organic phase was collected and vacuum-dried. Residues were resuspended in mobile phase and analyzed by HPLC-UV.

Pharmacokinetics and biodistribution assays. BNZ pharmacokinetic parameters were obtained from the plasma concentration-versus-time curve and calculated by application of the monocompartmental model.

The elimination (k_{el}) and absorption (k_a) constants were determined by the graphical method. The elimination ($t_{1/2b}$) and absorption ($t_{1/2a}$) half-lives were calculated as 0.693/*k*. The area under the curve from 0 to 6 h (AUC_{0-6}) was calculated by the trapezoidal method, and the area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-6} + (C_n/k_{el})$, where C_n is the last measurable concentration obtained at 6 h. The area under the first statistical moment curve from 0 to 6 h ($AUMC_{0-6}$) was calculated as $\{[(C_1 \times t_1) + (C_2 \times t_2)]/2\} \times (t_2 - t_1)$, where *t* and *C* are time and BNZ concentration, respectively. The area under the first statistical moment curve extrapolated to infinity ($AUMC_{0-\infty}$) was calculated as $AUMC_{0-6} + [(C_n \times t_n)/k_{el}] + (C_n/k_{el}^2)$, where C_n is the BNZ concentration obtained at the last measure and t_n is the time at last measure. The mean residence time (MRT) was calculated as $AUMC_{0-\infty}/AUC_{0-\infty}$. The clearance (CL) was calculated as dose/$AUC_{0-\infty}$, and the volume of distribution (*V*) was calculated as CL/k_{el}. The maximum concentration (C_{max}) and time to reach the maximum concentration (T_{max}) were obtained directly from the graph. All pharmacokinetic parameters were expressed as means ± standard deviations. In addition, the relationship between plasma and tissue AUC_{0-6} was determined as $AUC_{0-6,\ tissue}/AUC_{0-6,\ plasma} \times 100$ for a better evaluation of the BNZ amount in each individual organ.

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