Liposomal Benznidazole: A High-Performance Liquid Chromatographic Determination for Biodistribution Studies

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Abstract

In this work, an isocratic high-performance liquid chromatographic method for quantitation of liposomal benznidazole (BNZ) in biological tissues is presented. The method comprises protein precipitation together with an efficient extraction of bulk or liposomal BNZ with acetonitrile–dimethylsulfoxide (1:1, v/v) at a 2:1 (extraction solvent–tissue matrix, v/v or /vw) ratio; the process is completed by a final precipitation with trichloroacetic acid. The resultant supernatants are assayed chromatographically using a Kromasil C_{18} (25- × 0.4-cm i.d., 100 Å, 5-µm particle size), with an isocratic mobil phase consisting of acetonitrile–water (40:60, v/v), a flow rate of 0.9 mL/min, and detected at 324 nm. Bulk BNZ is used as a reference standard for the analysis of samples containing liposomal BNZ. The assay is linear over a concentration range of 0.75 (the lowest quantity of analyte determined with precision and accuracy of ≤ 20%) to 25 µg/mL-g in all liquid and solid matrices. Within-day precision is better than 6.4% in plasma and 8.6% in liver, the same for the two assayed concentrations. Between-day precision is 5.4% and 12.3% in plasma and 9% and 6.9% in liver for the two assayed concentrations, respectively. The absolute recoveries range between 70% and 97%. Therefore, the method is accurate and precise to be employed for detection of minor quantities of liposomal BNZ in biological tissues.

Introduction

American Trypanosomiasis (Chagas' disease) is an endemic disease affecting at least 20 million people in Latin America. More than 40 million are living under conditions in which they may be bitten by the vector insect infected with the parasite (1). Benznidazole [*N*-benzyl-(2-nitroimidazoyl) acetamide] (BNZ) is the only approved drug for the chemotherapy of Chagas' disease in Argentina. BNZ's metabolization is mediated by nitroreductases, which produce free radicals as intermediate products; those free radicals form adducts with parasite's macromolecules. This mechanism is the origin of the drug's toxicity for the parasite and the same radicals also produce serious side effects in the host (2). Despite the major importance of the problem, to date, no available treatment is satisfactory because BNZ-based therapies do not achieve a parasitologic cure (3).

The incorporation of a drug into a liposomal carrier is a well-known and powerful tool to modify both its pharmacokinetics and biodistribution (4,5). The encapsulated drug is forced to reach a specific tissue or cell type, avoiding unnecessary degradation/consumption in nondesired sites. As a consequence, the toxicity of the drug is reduced, whereas its activity is enhanced, as it has been demonstrated for liposomal amphotericin B (6) and nystatin (7). With the purpose of increasing the amount of BNZ delivered to infected Kupffer cells, which naturally phagocyte multilamellar liposomes, the authors have recently developed multilamellar liposomes composed of hydrogenated soybean phosphatidylcholine (HSPC), distearoyl-phosphatidylglycerol (DSPG), and cholesterol (Chol) loaded with BNZ (8). In this paper, a method for quantitating liposomal BNZ in biological environments is described. The method is composed of a liquid–liquid extraction followed by high-performance liquid chromatography (HPLC) separation and UV detection. The validation of this method is described, and examples of the application to biodistributive studies are given.

Experimental

Materials

BNZ (99.85% purity) was supplied by Roche Argentina (RO-07-1051/000) (Buenos Aires, Argentina). HSPC and DSPG were from Northern Lipids (Vancouver, Canada) Chol and Tris buffer were from Sigma (St. Louis MO). Normal blood, plasma, and tissues were obtained from untreated control rats. Dimethyl sulfoxide (DMSO) and acetonitrile (ACN) were of HPLC quality (Carlo Erba Reagenti, Milan, Italy). Trichloroacetic acid (TCA) was analysis grade from Anedra (Buenos Aires, Argentina).

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Chromatographic system

HPLC was performed on Beckman System Gold (Beckman Instruments, Fullerton, CA) with a programmable Solvent Module 125 and a programmable detector Module 166 (Software System Gold, the Personal Chromatograph Digital Research, Beckman). The wavelength was set at 324 nm (the maximun absoption wavelength of BNZ in the mobile phase), with a response time of 1 s. The analytical column was Kromasil C_{18} (Teknokroma, Madrid, Spain) (25- × 0.4-cm i.d., 100-Å, 5-µm particle size) and was run at room temperature (20ºC). The mobile phase used was acetonitrile–water (40:60, v/v) degassed by vacuum. Elution was performed isocratically at a flow rate of 0.9 mL/min, with a sample injection volume of 20 µL.

Liposomal preparation

Multilamellar liposomal benznidazole (MLV-BNZ) composed of HSPC–DSPG–Chol (2:1:2, mol/mol) was prepared by mixing lipids from $C_{l3}CH$–CH_3OH (9:1, v/v) solutions and BNZ in DMSO solution at a 5:100 (mol/mol) drug-to-lipid ratio. The lipid–BNZ mixture was rotary evaporated at 50ºC in round-bottom flasks until organic solvent elimination. The resulting film was flushed with N_2 and suspended in 500 µL of 10mM Tris–HCl buffer (pH 7.4) up to a final concentration of approximately 20–30 µmol/mL total lipids. The free BNZ was eliminated by one step of centrifugation, and the liposome pellet was resuspended in Tris buffer. The liposomal lipid concentration was determined by a colorimetric phosphate microassay (9), and the liposomal BNZ content determination by HPLC was performed by disrupting the liposome and extracting the BNZ in a ninefold volume excess of DMSO. A 20-µL volume of the extract was then injected into the column; the quantitation was based on the peak area–concentration response of a calibration curve performed with bulk BNZ in DMSO. No interference with lipids was checked.

Preparation of standard solutions

A stock standard solution of BNZ (14 mg/mL) was prepared by weighting the appropriate amount of bulk BNZ and dissolving it in DMSO. Further stock solutions (1.4 mg/mL and 140 and 28 µg/mL) were made by diluting the initial stock standard s olution in DMSO. Separate stock standard solutions were prepared for standard curves and quality control samples, respectively. The stock solutions were protected from light and stored at –20ºC.

Sample preparation

Liquid and liquid tissues in matrices

The liquid and liquid tissues matrices were composed of 10mM Tris buffer (pH 7.4) and blood and plasma obtained after 10 min at 1500 g blood centrifugation, respectively. Extraction of bulk and liposomal BNZ from matrices was achieved by the addition of 400 µL ACN–DMSO (1:1, v/v) to 200 µL of sample at a 2:1 (extraction solvent–matrix, v/v) ratio and incubated at 4ºC for 30 min in 1.5-mL polypropylene tubes. After 20 min of centrifugation at 6000 g, supernatants were transferred to fresh tubes, incubated 15 min at –12ºC with 100 µL of TCA solution (10%, w/v) (to favor the protein precipitation), and centrifuged 10 min at 10.000 g. Finally, the supernatants were transferred to new tubes and 20 µL injected to the column.

Solid tissues matrices

Liver, spleen, kidney, heart, and lungs were obtained from non-injected rats, washed with Tris buffer in order to remove all the blood, minced in small pieces, and stored at –80ºC until use. A portion of each matrix was thawed, weighted 150–200 mg, and diluted with 100 µL of deionized water. The homogenization–extraction of bulk and liposomal BNZ was performed by the addition of CAN–DMSO (1:1, v/v) at a 2:1 (extraction solvent–matrix, w/w) ratio. The process was completed following the same steps as described for liquid and liquid tissues matrices, and 20 µL was injected to the column.

Standard curves and quality control samples

Liquid matrix

A seven-point calibration curve ranging from 0.75 to 25 µg BNZ/mL was prepared by adding 5–30 µL of BNZ from a stock solution in DMSO to 200 µL of Tris buffer (10mM, pH 7.4).

Liquid tissues matrices

A seven-point calibration curve ranging from 0.75 to 25 µg BNZ/mL was prepared by adding 5–30 µL of BNZ from stock solution in DMSO to 200 µL of rat blood or plasma. Two points for quality control of the calibration curve were prepared in similar fashion to determine the precision and accuracy of the assay.

Solid tissues matrices

A seven-point calibration curve ranging from 0.75 to 25 µg BNZ/g was prepared by adding 5–30 µL of BNZ stock solution in DMSO to 200 mg rat solid tissue matrices. Two points for quality control of the calibration curve were prepared in similar fashion to determine the precision and accuracy of the assay.

Controls

Controls (without BNZ) for liquid and solid tissue matrices were extracted as described in the Sample preparation section. Two points of each calibration curve for all matrices were prepared by adding 3.5 and 20 µg/mL-g BNZ from liposomal BNZ in a range of 0.1–3 mg total lipid.

After the addition of bulk or liposomal BNZ to the calibration curves for all matrices, as well for quality and matrices controls, the samples were homogenized–extracted as previously described in the Sample preparation section.

Validation

Duplicated calibration curves were analyzed on each of three days together with sextuplicates of the lower and higher concentration points. Controls for liquid and tissue matrices and quality controls were analyzed together with each calibration curve. The calibration curves were obtained by least-squares linear regression analysis of the peak areas versus the concentration of the compound. Quantitation in the different matrices was based on the peak area–concentration response of the calibration curves.

Biodistribution experiments

Six female rats weighing 170–180 g received a dose of 0.2 mg BNZ/kg body weight of bulk BNZ (dilution of BNZ from DMSO in Tris 10mM buffer, pH 7.4; the amount of DMSO in the final solution did not exceeded 2%) or liposomal BNZ intravenously via the

tail vein. The animals were sacrificed 60 min post injection. Blood samples were collected on tubes containing sodium citrate; liver, spleen, lungs, heart, and kidney were collected, washed, weighed, and stored at –80ºC.

Results and Discussion

Traditional quantitation of bulk BNZ (drug structure shown in Figure 1) by HPLC in mice tissues has already been performed by Walton and Workman (10,11), when investigating its use as chemosensitizer. In their work, the bulk drug doses are in the order of 650 mg of BNZ/kg body weight. In the authors' situation, liposomal doses are mainly limited by the drug–lipid ratio, which is characteristic of each liposomal formulation and by the lipid concentration of the liposomal suspension. In case of low drug–lipid ratio formulations (as liposomal BNZ is), doses are frequently limited by the maximum lipid dose/kg body weight tolerated by rats (12). According to this, liposomal doses should not be higher than 0.2–1 mg BNZ/kg body weight. Therefore, the plasma and tissue levels of liposomal drug are more than 3200-fold lower than those expected in bulk BNZ biodistribution studies. Regarding its potential medical use, the authors' faced the development of a new extraction and quantitation method to be used in future testing of liposomal BNZ biodistribution.

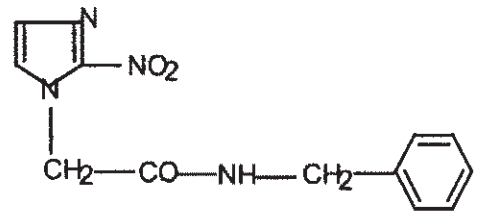

Figure 1. BNZ structure.

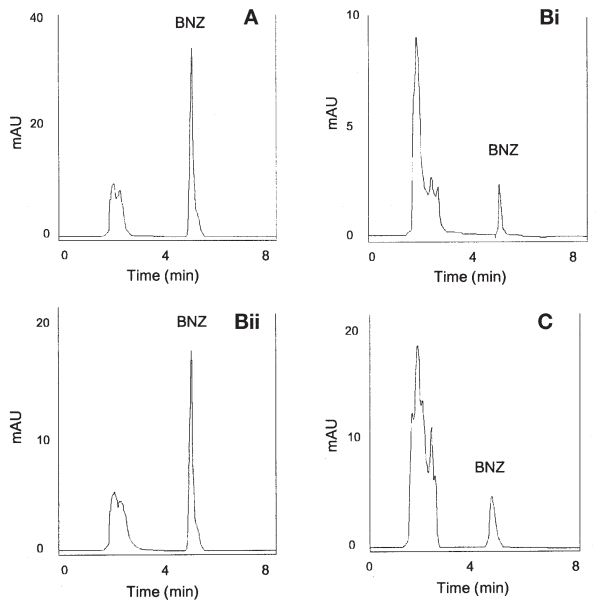

Figure 2. Typical chromatograms of supernatants extracted from different matrices spiked with bulk BNZ: (A) liquid matrix, (B) liquid tissue matrix (blood) spiked with bulk BNZ at concentrations [(Bi) 3.5 and (Bii) 20 µg/mL], and (C) solid tissue matrix (liver). The mean retention time of the BNZ peak was 4.7–4.8 min. There were no differences in the appearance of the chromatograms between blood and plasma (data not shown).

Separation

The original method to extract BNZ from liquid and solid tissue matrices involved the use of methanol (MeOH) (9). However, in the authors' experience, MeOH was found unsuitable to quantitatively extract from liquid and solid tissues the low drug amount injected as liposomal BNZ. Thus, several solvents were assayed and ACN–DMSO (1:1, vol/vol) was finally selected because the resulting chromatograms presented the highest signal/noise ratio (s/n) and BNZ peaks were best resolved from residual matrix signals. The solubility of BNZ in DMSO was considerably higher than in MeOH (more than tenfold); liposomes were satisfactorily dissolved in ACN–DMSO, and the ACN content of the extraction mixture was high enough to precipitate more than 99% of matrix proteins in the first centrifugation step. The further addition of TCA solution improved the chromatographic profile by eliminating residual interfering peaks. In vitro and in vivo drug assays were performed, and the resulting chromatograms are described as follow:

In vitro assays

Figure 2A–C shows chromatograms corresponding to bulk BNZ in different matrices. In there, the absence of matrix-inter-

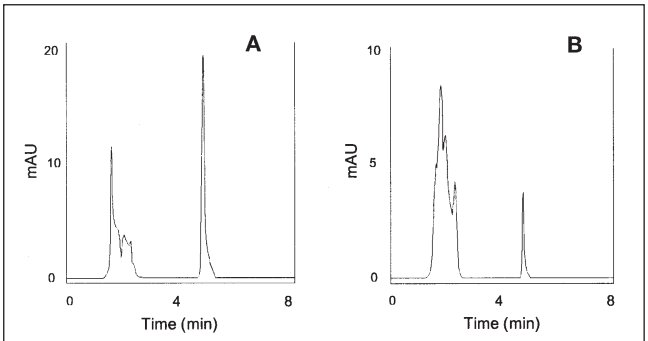

Figure 3. Typical chromatograms of supernatants extracted from different matrices spiked with liposomal BNZ: (A) liquid tissue matrix (blood, 20 µg/mL) and (B) solid tissue matrix (liver, 5 µg/g).

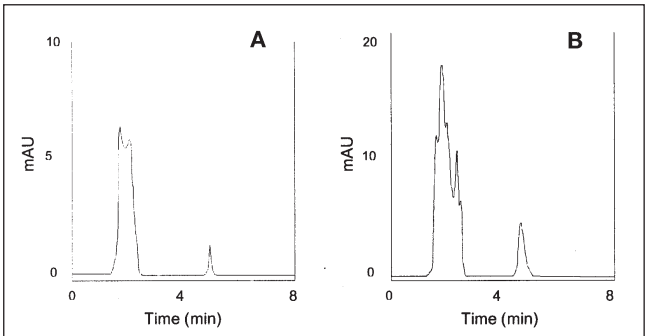

Figure 4. Chromatograms of supernatants extracted from: (A) blood and (B) liver after 60 min of intravenous injection of liposomal BNZ in a dose of 0.2 mg BNZ/kg.

fering peaks with BNZ's signal can be seen. Figure 3A and 3B corresponds to chromatograms of liquid and solid tissue matrices (blood and liver) spiked with liposomal BNZ. Shown is that liposomal lipids do not alter the BNZ's time of retention.

In vivo assays

Figure 4A and 4B show chromatograms corresponding to the detection of BNZ in liquid and solid tissue matrices (blood and liver) 60 min after intravenous tail injection of liposomal BNZ.

Specificity

To assess the validity of using bulk BNZ as reference standard for the analysis of samples containing liposomal BNZ, normal rat blood (n = 3), plasma (n = 3), and liver (n = 3) were spiked with liposomal BNZ up to a final concentration of 3.5 and 20 µg/mL-g. All the samples were extracted and quantitated using the calibration curve prepared from bulk BNZ. Accuracies at the two concentrations were +4.25% and –9% of the expected value, and precision as assessed by the coefficient of variation (CV) of the mean was 5.7% and 13% for the blood sample; accuracies for plasma were 9% and 7.54% with a CV of 14% and 0.75%; and accuracies for the liver were 5% and 8.5% with a CV of 9% and 11%.

Table I. Calibration Curve Parameters from Different Matrices

Matrix		Slope (SD*, n = 5)	Intercept (SD, n = 5)	Correlation coefficient
Liquid		18.85 (0.460)	–0.025 (0.01)	0.998
Liquid tissues	Blood	18.32 (0.452)	–0.006 (0.001)	0.997
	Plasma	15.89 (0.422)	–0.030 (0.01)	0.996
Solid tissues	Liver	21.24 (0.616)	0.017 (0.005)	0.997
	Spleen	12.66 (0.592)	0.004 (0.001)	0.995
	Lung	16.76 (0.420)	–0.018 (0.005)	0.996

* SD = standard deviation.

Table II. Within-Day Variations of the Assay in Liquid (Plasma) and Solid (Liver) Tissue Matrices*

Tissue matrix	Spike concentration (µg/mL)	Mean calculated concentration (µg/mL)	SD	CV (%)	Accuracy (%)
Liquid	0.75	1.05	0.06	6.31	+15.29
		1.20	0.06	5.67	+20.36
		1.05	0.001	1.30	+16.33
	25	24.12	1.30	6.09	–1.55
		25.58	0.98	2.08	–25.27
		26.31	1.20	6.44	–27.18
Solid	0.75	1.05	0.07	6.75	+13.65
		1.05	0.04	4.28	+11.2
		1.21	0.06	5.39	+15.42
	25	28.26	1.98	7.08	+11.28
		29.97	2.55	8.65	+16.38
		29.40	0.99	3.60	+14.89

* Each concentration was prepared six times (n = 6).

Linearity and precision

Table I shows calibration curve parameters for different matrices, all of which had correlation coefficients higher than 0.99. The assay was linear over the concentration range of 0.75–25 µg/mL-g.

Within-day precision, as measured by the CV of the daily mean (n = 6), and accuracy (Table II) showed that the precision was better than 6.4% for the two concentration assays for plasma and 8.6% for rat liver.

Between-day or overall precision presented in Table III shows a CV of 5.4% and 12.3% (n = 18) for plasma and values of 9% and 6.9% (n = 18) for liver.

Lower limit of quantitation

The lower limit of quantitation (LOQ), 0.75 µg/mL-g, was defined as the ratio s/n = 10. Six replicates of the lowest standard concentration were analyzed to evaluate the LOQ. At the LOQ, the percent variation of the measured concentrations was 5%, and the deviation of the mean of the measured concentrations from their nominal value was 18%.

Absolute recoveries

The absolute recoveries of bulk BNZ and liposomal BNZ from the liquid tissue and solid tissue matrices were determined by dividing the slopes of the calibration standard curves in each matrix by the slope of the calibration standard of bulk BNZ in the liquid matrix (13). The absolute recoveries were 94% for blood, 86% for plasma, 97% for liver, 70% for spleen, and 90% for lungs.

Biodistribution

The results shown in Table IV indicate that liver, lungs, and kidneys (the last two organs having extensive capillary beads in which high-sized liposomes are kept trapped) were the main targets for liposomal BNZ. Lower blood levels after 60 min in circulation, with absence of BNZ in plasma together with complete association to red cells were achieved by liposomal BNZ. Oppositely, bulk BNZ mainly remained in circulation, principally associated to plasma fraction, and the percentage of liver uptake was almost half of that achieved by liposomal BNZ. These results showed liposomal and bulk BNZ as two different drugs because both presented a differential biodistribution when intravenously injected at the same dose.

Table III. Between-Day Variations of the Assay in Liquid (Plasma) and Solid (Liver) Tissue Matrices*

Matrix	Spike concentration (µg/mL)	Mean calculated concentration (µg/mL)	SD	CV (%)	Accuracy (%)
Liquid tissue Matrix	0.75	1.05	0.06	5.39	+13.48
	25	25.58	1.23	12.3	+16.21
Solid tissue Matrix	0.75	1.05	0.10	9.03	+16.56
	25	29.26	1.98	6.87	+18.96

* Each concentration was prepared six times (n = 6) and measured for three consecutive days.

Table IV. Percentages of the Injected Doses Found in Each Organ after 1 h of Injection*

Injection	Plasma	Red cells	Liver	Lungs	Spleen	Kidneys	Heart	Tail
Bulk BNZ	78	9.1	9.1	1.42	nd†	0.63	nd	0.08
Liposomal BNZ	0	65	16.8	7.45	0.81	9.85	nd	1.44

* Three rats were injected per treatment, and three aliquots of each organ were submitted to extraction and quantitation assay.
† nd = not detectable.

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Conclusion

The assay procedure presented in this report provides a simple and rapid means for the accurate and precise quantitation of liposomal BNZ. The method can be used for the analysis of BNZ in tissues and blood from rats and humans.

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