Bioorganic & Medicinal Chemistry 16 (2008) 7622–7630

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Determination and characterization of new benzimidazoles with activity against *Trypanosoma cruzi* by UV spectroscopy and HPLC

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ARTICLE INFO

Article history:
Received 9 June 2008
Revised 30 June 2008
Accepted 6 July 2008
Available online 13 July 2008

Keywords:
pK_a
log *P*
HPLC
UV
Determination
Antichagasic
Nitrophenylbenzimidazole

ABSTRACT

This work presents the development of analytical methodologies by UV spectrophotometry and HPLC to characterize five nitroarylbenzimidazole derivatives with activity against *Trypanosoma cruzi*: NB, BNB, PNB, PMNB and PCNB. Both methodologies exhibit adequate repeatabilities and reproducibilities (CV < 2%) and recoveries higher than 98%. The ionization constants (pK_a), lipophilicity (log *P*) and effective permeability (Pe) are reported. The five compounds present an inhibitory effect on the *T. cruzi* growth (epimastigotes) at 1–100 μM concentration range in an order rank of PMNB > PCNB > PNB > BNB > NB. Additionally, cyclic voltammetric data reveal that the nitroarylbenzimidazole derivatives might sustain their effects on growth and oxygen uptake on *T. cruzi* epimastigotes.

1. Introduction

Benzimidazole is a very important pharmacophore in drug discovery, and its derivatives are an important class of bioactive molecules in the field of drugs and pharmaceuticals.[1,2] Some of them exhibit significant activity against several viruses, and have demonstrated to be potent antiparasitic agents antitumour agents, antimicrobial agents, and inhibitors of the hepatitis C virus RNA polymerase.[3–6] Benzimidazoles are also veterinary drugs widely used for prevention and treatment of parasitic infections in agriculture and aquaculture. In addition, some of them also have applications as pre- or post-harvest fungicides for the control of a wide range of fungi, which affect field crops, stored fruit and vegetables.[7]

On the other hand, nitroaromatic compounds are a very important group, which have been used extensively in the treatment of anaerobic infections, and are under continuum investigation. Regarding their use in cancer therapy, nitroaromatic compounds are recognized by acting as specific cytotoxins and markers for hypoxic regions in tumours. There is a direct proof that free-radical metabolites are involved in many applications including important antitumuor and antiparasitic agents.[8,9] Furthermore, nitrogroup bearing compounds are considered as possible leads for the development of drugs against Chagas' disease.[10,11] In this issue, there is good evidence that some electrochemical properties of nitro compounds, as its reduction potential, can be correlated with the pharmacological effects of these compounds.[8,12]

In consequence, by the combination of a nitroaromatic ring to a benzimidazole nucleus, substances of relevant biological activity against cells of parasites and/or animals might be obtained, with the aim to find potential drugs useful in a diverse range of pathologies.

Parasitic diseases such as malaria, leishmaniasis and trypanosomiasis represent a significant global burden and propose a great challenge to drug discovery, due to their intracellular nature and disseminated locations. Because of the poor rate in the discovery in the anti-parasitic segment seen in the last few decades, an effective management of existing drugs by modulating their delivery is necessary.[13] The only trypanocidal chemotherapies available for Chagas' disease are solid dosage forms (nifurtimox and benznidazole), but they have disadvantages associated with poor bioavailability. Moreover, drugs as nifurtimox have significant side effects, including anorexia, vomiting, peripheral polyneuropathy, depression of bone marrow, and allergic dermopathy.[14] As a consequence of these adverse reactions, frequently oral treatments have to be discontinued. If the pharmacokinetics of the drug improves, the therapeutic dose is expected to decrease, with the consequent diminution of side effects. The reasons for the pronounced differ-

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doi:10.1016/j.bmc.2008.07.021

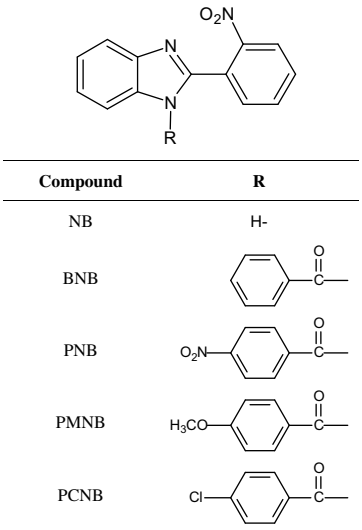

Figure 1. Chemical structures of 2-(2-nitrophenyl)-1H-benzimidazole (NB), 1-benzoyl-2-(2-nitrophenyl)-1H-benzimidazole (BNB), 1-(4-nitrobenzoyl)-2-(2-nitrophenyl)-1H-benzimidazole (PNB), 1-(4-methoxybenzoyl)-2-(2-nitrophenyl)-1H-benzimidazole (PMNB) and 1-(4-chlorobenzoyl)-2-(2-nitrophenyl)-1H-benzimidazole (PCNB).

ence in the antiparasitic efficacy of nitro heterocyclic compounds in acute and chronic stages of the disease are not yet understood, but they could be related to unfavourable pharmacokinetic properties of the drugs in the chronic stages.[14]

Physicochemical properties as log P value, stability and ionization constants are crucial for drug delivery and biological activity of drugs. Both nifurtimox and benznidazole present low log P values (1.2 and −0.34, respectively), and nifurtimox undergoes rapid biotransformation, possibly due to a presystemic first-pass effect, generating several unidentified metabolites.[10,15] In consequence, the design of new stable drugs with higher log P could improve the pharmacokinetics properties.[16–18]

In this paper, we present the development of analytical methodologies by UV-spectrophotometry and HPLC to study physicochemical properties as ionization constants, permeability and antiparasitary activity of five nitroarylbenzimidazole derivatives: 2-(2-nitrophenyl)-1H-benzimidazole (NB), 1-benzoyl-2-(2-nitrophenyl)-1H-benzimidazole (BNB), 1-(4-nitrobenzoyl)-2-(2-nitrophenyl)-1H-benzimidazole (PNB), 1-(4-methoxybenzoyl)-2-(2-nitrophenyl)-1H-benzimidazole (PMNB) and 1-(4-chlorobenzoyl)-2-(2-nitrophenyl)-1H-benzimidazole (PCNB) (Fig. 1). Also, the cyclic voltammetric behaviour of NB, BNB, PNB, PMNB and PCNB is reported.

2. Results and discussion

2.1. Analytical studies

In Figure 2, NB, BNB, PNB, PMNB and PCNB UV–Vis spectra at different pH are shown. As can be seen in Figure 2A, NB exhibits two maximums at 226 and 270 nm, and in alkaline pH the wave at 270 nm undergoes a bathochromic shifting at 288 nm. In addition, NB exhibited two isosbestic points at 254 and 283 nm.

On the other hand, BNB exhibits two maximums in acid pH at 223 and 253 nm, and the band at 223 nm undergoes a hypochromic shift at alkaline pH (Fig. 2B). Similar behaviour was observed for PNB, which exhibits two maximums at 230 and 263 nm, and, in alkaline pH, the band at 230 nm undergoes a hypochromic shifting (Fig. 2C).

Additionally, PMNB displays two maximum at 230 and 263 nm, and an upper shoulder at 297 nm. In alkaline pH, the waves at 228 and 297 nm undergo a hypochromic shifting (Fig. 2D). In Figure 2E, PCNB exhibits two maximum at 228 and 258 nm and, in alkaline pH, the band at 228 nm undergoes a hypochromic shifting.

In order to develop an analytical UV–Vis method, NB and BNB were diluted in ethanol/Britton-Robison buffer (30/70) (at pH 3 and 5, respectively). In parallel, PNB, PMNB and PCNB were diluted in acetonitrile/Britton–Robinson buffer (20/80) at pH 6.0 for each compound. Furthermore, molar absorptivity values of 15,900 (270 nm), 24,000 (253 nm), 25,000 (263 nm), 25,887 (263 nm) and 26,173 (258 nm) for NB, BNB, PNB, PMNB and PCNB, respectively, were calculated.

Alternatively, with the purpose to develop a HPLC method, system suitability tests in the presence of the corresponding degradation and precursor products of each compound were carried out.[19]

According to the gradient system established for NB, it exhibited Rt = 8.356 ± 0.102 min, k' = 3.46 and R = 1.05. In the accelerated degradation studies, NB was stable to the hydrolysis, and produces one photolytic product. Using the isocratic system selected for BNB, it displayed Rt = 7.117 ± 0.097 min, k' = 4.30 and R = 7.82. In parallel, the isocratic system selected for PNB generated a retention time of 6.185 ± 0.081. In the accelerated degradation studies, BNB produces NB as hydrolytic product, and PNB generates NB and *p*-nitrobenzoate as hydrolytic products.

Regarding the gradient system composed for PMNB, it exhibited a retention time of 7.93 ± 0.087 min. In the accelerated degradation studies, PMNB generates NB and *p*-methoxybenzoate as hydrolytic products. For PCNB an isocratic elution was composed. Under those conditions, it displayed Rt = 6.89 ± 0.06 min, k' = 3.58 and R = 21.03. In the accelerated degradation studies, PCNB generates NB and *p*-chlorobenzoate as hydrolytic products. In Figure 3, typical chromatograms in the optimal conditions for each compound are shown.

Furthermore, with the aim of evaluating the selectivity before possible interferences owing to common tablet excipients, recovery studies were done. In all cases, recovery percentages over 98.5% were found. The analytical performance parameters for UV and HPLC are summarized in Table 1.

2.2. Application and characterizations

From the studied compounds behaviour with pH variations on spectroscopy (Fig. 2), data wavelengths of 288 and 226 nm for NB, 223 nm for BNB, 230 nm for PNB, 230 nm for PMNB and 228 nm for PCNB were selected to calculate each compound pK_a value, since at that wavelengths exist a high sensitivity zone to pH variations. NB is an amphotheric molecule and exhibits pK_{a1} = 5.69 ± 0.06 corresponding to a weak base of $N-1$ benzimidazole ring (N=C) and pK_{a2} = 11.38 ± 0.03 corresponding to an acid proton of $N-3$ benzimidazole ring (N–H). The other four compounds display only one pK_a value related to the imidazole nitrogen weak base. The pK_a values for BNB, PNB, PMNB and PCNB are 4.90 ± 0.02, 4.79 ± 0.04, 4.71 ± 0.04 and 4.69 ± 0.04, respectively (Fig. 4).

Using HPLC, the partition coefficients ($\log P$) of each compound were determined.[20,21] By plotting the $\log P$ values against $\log[(t_R - t_0)/t_0]$ ($\log k$) of the known compounds, where t_0 was 0.987 min as determined by using potassium nitrate, the following calibration curve was obtained:

$$\log P = 1.69 \log k + 2.69 \quad (r = 0.995,\ n = 6).$$

NB, BNB, PNB, PMNB and PCNB exhibited $\log k$ values of −0.285, +0.224, +0.047, +0.276 and +0.503, in that order, and the distribution coefficients of the neutral molecules expressed as $\log P$ values were 2.2, 3.1, 3.2, 2.8 and 3.6, respectively. These high values could be due to the higher permeability through biological barriers *in vivo* of these compounds with respect to nifurtimox and benznidazole.[18]

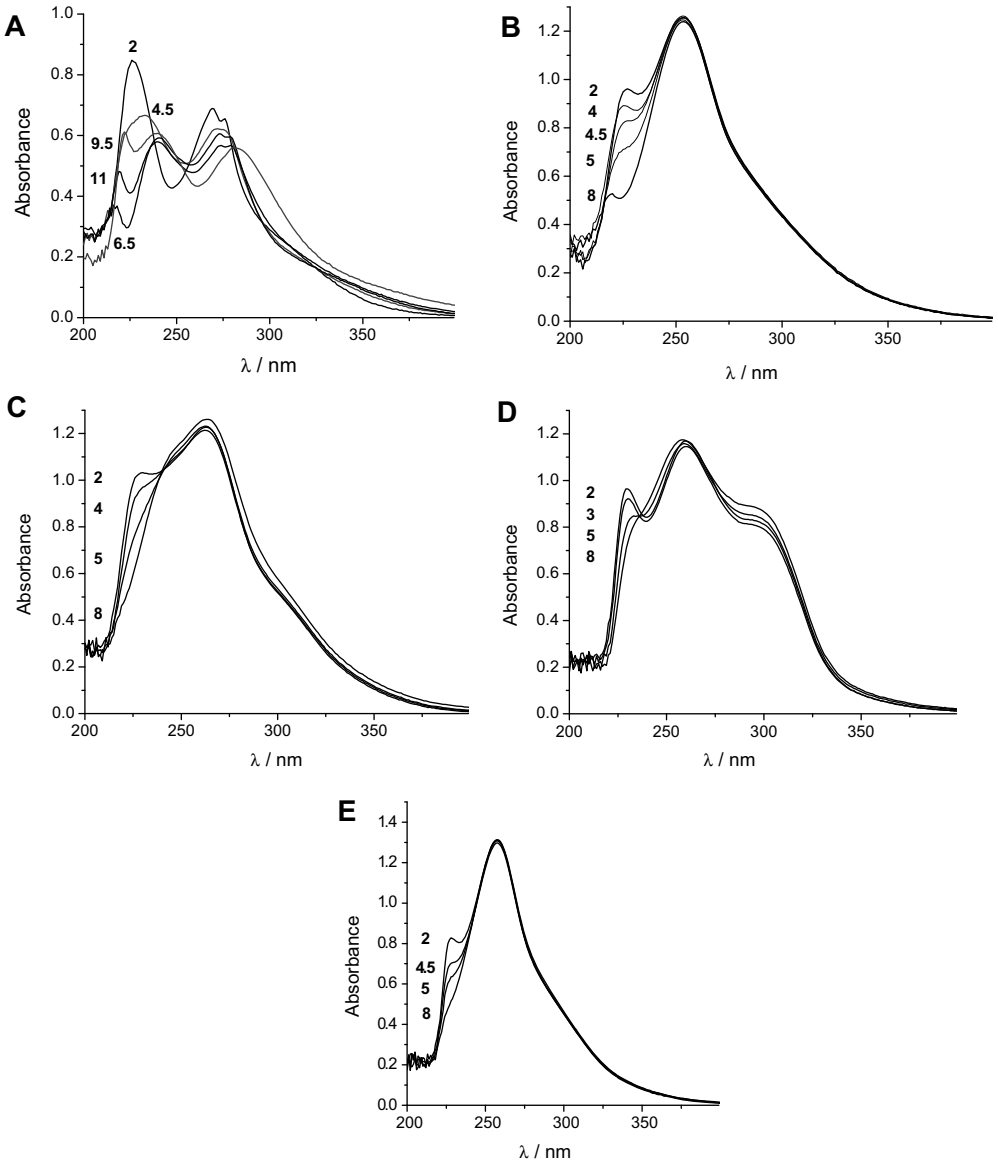

Figure 2. Evolution of UV–Vis spectra of NB (A), BNB (B), PNB (C), PMNB (D) and PCNB (E) with pH (NB and BNB in ethanol/0.1 M Britton–Robinson buffer 30/70, PNB, PMNB and PCNB in acetonitrile/0.1 M Britton–Robinson buffer 20/80).

By using HPLC and UV–Vis spectrophotometry methodologies, effective permeability of NB was determined. In *Parallel Artificial Membrane Permeability Assay*, NB exhibited Pe values of 0.8×10^{-5} cm/seg (HPLC) and 1.2×10^{-5} cm/seg (UV), indicating that NB could have a passive transport in the employed model. On the other hand, due to the high log*P* value of BNB and PCNB previously determined, their corresponding Pe could not be calculated by this method, because the compounds remain retained in the membrane at all assayed concentrations. Besides, both PNB and PMNB were not dissolved under the required assay conditions.

With an eye towards this the studied compounds may produce their biological action by bioreduction, such as nifurtimox and other nitroderivatives, cyclic voltammetric experiments were carried out.[8,22] From these experiments in aprotic media (at 1 V/s), NB, BNB, PNB, PMNB and PCNB exhibited cathodic potentials of −715, −899, −936 and −1110, −700 and −650 mV, respectively (Fig. 5). Taking into account that nifurtimox exhibits a cathodic potential of −876 mV in the same experimental conditions, these values obtained for NB, BNB, PNB, PMNB and PCNB could bear witness to the effects both on growth and on oxygen uptake on *T. cruzi* epimastigotes.[23]

2.3. Biological studies

To determine the inhibitory ability of the studied compounds, we investigated their effect on epimastigote growth in culture by nephelometry and respiration by polarography. NB, BNB, PNB, PMNB and PCNB present an inhibitory effect on the *T. cruzi* growth (epimastigotes) at 100 μM concentration range, compared with growth control (Fig. 6).

According to the obtained results, PMNB and PCNB possess higher inhibitory activity than PNB, BNB and NB at all the tested concentrations. Furthermore, all compounds at 100 μM concentration exhibit a similar effect at 10 μM nifurtimox concentration.[10,11,24]

BNB disables the growth of *T. cruzi* epimastigotes (MF strain) at IC_{50} = 94 μM, which is 6–9-folds higher than nifurtimox (10–15 μM according to strain); whereas NB inhibits the growth at IC_{50} = 117 μM, which is 8–11-folds higher than nifurtimox.[11] The

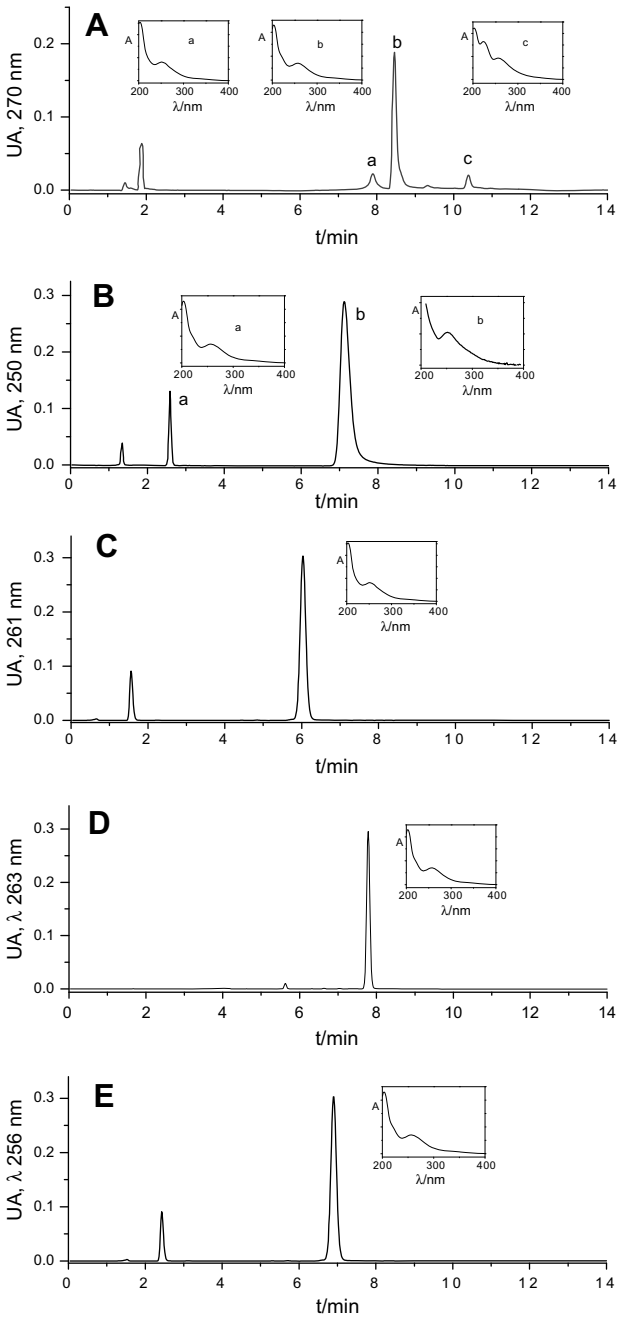

Figure 3. Typical chromatograms of NB (A), BNB (B), PNB (C), PMNB (D) and PCNB (E) in the optimal experimental conditions from selectivity assay. In A (a) nitrobenzaldehyde, (b) NB, (c) photolysis product. In B: (a) NB (from hydrolysis trial), (b) BNB. UV spectrum of each signal inserted.

IC_{50} value for PNB corresponds to 80 μM, whereas the IC_{50} for PMNB and PCNB are 64 μM (4–6-folds higher than nifurtimox) and 78 μM (5–8-folds higher than nifurtimox), respectively. These results are shown in Table 2.

Additionally, when oxygen uptake experiments on *T. cruzi* epimastigotes were carried out, BNB exhibited a higher inhibition than NB, with an oxygen uptake decreasing 40% and 20% for 300 μM of NB and BNB, respectively (data not shown).

3. Conclusions

Although NB, BNB, PNB, PMNB and PCNB presented lower biological activity than nifurtimox, the presence of benzimidazole and nitroaromatic groups in the same molecule confers higher log*P* values to NB, BNB, PNB, PMNB and PCNB with respect to nifurtimox and benznidazole, this characteristic being an important parameter for drug permeability. The log P and permeability assay results for BNB, PNB, PMNB and PCNB are consistent with the fact that at pH 7.4 they remain mainly as non-ionized form (according to their pK_a values). Furthermore, the obtained results in the biological assays may be due to a higher penetration into the parasite of PMNB and PCNB. However, PNB (which contains two nitrogroups) did not have a higher inhibitory activity than PMNB and PCNB, thus it is probable that the membrane permeability owing to their lipophilicity may be more important for the compound activity against *T. cruzi* than its reduction potential. In turn, it is possible that the PNB second reduction potential value might be lower than necessary to enhance its potency as antichagasic agent, and hence modifying the ubication of this second nitrogroup (to produce a reduction potential between −700 and −900 mV) might be useful for this feature. In consequence, the lipophilicity (from the benzimidazole moiety), along with the reduction potential (produced by the nitrogroup present in the molecule), both are essential for the studied compounds biological activity.

Regarding the results from the biological assays performed to NB, BNB, PNB, PMNB and PCNB, in spite of their lower antichagasic activity *versus* nifurtimox, subsequent studies of these compounds are necessary, because if they present lower toxicity and higher bioavailability compared with nifurtimox, they might be good candidates for use in Chagas' disease therapy.

In conclusion, the compounds characterized in this work are potential, and are promising leads for the design of new stable, active and permeable drugs for treatment of Chagas' disease, and to consider the potential use of these derivatives and new analogs, toxicity and further studies are currently in progress.

4. Experimental

4.1. Reagents and drugs

2-(2-Nitrophenyl)-benzimidazole (NB) ($C_{13}H_9N_3O_2$, 239.06 g/mol, 277–278.5 °C), 1-benzoyl-2-(2-nitrophenyl)-benzimidazole (BNB) ($C_{20}H_{13}N_3O_3$, 343.33 g/mol, 161 °C), 1-(4-nitrobenzoyl)-2-(2-nitrophenyl)-benzimidazole (PNB) ($C_{20}H_{12}N_4O_5$, 388.33 g/mol, 174–176.9 °C), 1-(4-methoxybenzoyl)-2-(2-nitrophenyl)-benzimidazole (PMNB) ($C_{21}H_{15}N_3O_4$, 373.36 g/mol, 124–126 °C) and 1-(4-chloro benzoyl)-2-(2-nitrophenyl)-benzimidazole (PCNB) ($C_{20}H_{12}N_3O_3Cl$, 377.78 g/mol, 127–129 °C) were obtained in our laboratory according to a procedure previously described.[25,26] The purity of these compounds was assessed by ^1H NMR, IR, ^{13}C NMR and melting point. All reagents were of analytical grade, unless indicated otherwise. Sodium hydrogen phosphate, phosphoric acid and acetonitrile HPLC grade were used. Deionized water was obtained in the laboratory, using ionic exchange columns (Milli-Q).

4.2. Solutions preparation

4.2.1. Buffer solutions

0.1 M Britton–Robinson buffer (acetic acid/boric acid/phosphoric acid)[27] for UV experiments was used, and pH was adjusted with concentrate solutions of NaOH or HCl. For HPLC, a 0.05 M buffer phosphate solution (di-sodium hydrogen phosphate anhydrous salt) adjusted at pH 4.5 with phosphoric acid was used.

4.2.2. Stock drug solutions

2.39 mg of NB and 3.43 mg of BNB were dissolved and diluted up to 10 mL with ethanol, besides 3.88 mg of PNB, 3.73 mg of PMNB and 3.78 mg of PCNB were dissolved in acetonitrile, to ob-

Table 1
Analytical parameters of *o*-nitrophenylbenzimidazole derivatives for UV and HPLC developed methods

Parameter	UV					HPLC				
	NB	BNB	PNB	PMNB	PCNB	NB	BNB	PNB	PMNB	PCNB
Wavelength detection (nm)	270	253	263	263	258	270	253	263	263	258
Repeatability, CV(%) 1×10^{-5}; 5×10^{-5}; 1×10^{-4} M	1.0; 0.3; 0.2	1.3; 1.3; 0.9	1.3; 1.2; 0.9	0.9; 0.3; 0.8	1.2; 1.2; 1.1	0.4; 0.3; 0.4	0.3; 0.4; 0.5	1.3; 1.5; 1.0	1.8; 1.3; 1.0	1.4; 1.0; 1.1
Reproducibility, CV(%) 1×10^{-5}; 5×10^{-5}; 1×10^{-4} M	1.2; 0.8; 0.4	1.6; 1.4; 1.1	1.5; 1.3; 1.1	1.2; 0.6; 1.2	1.4; 1.5; 1.6	0.5; 0.4; 0.5	0.4; 0.7; 0.6	1.6; 1.7; 1.3	1.9; 1.5; 1.6	1.7; 0.9; 1.3
Recovery (%) ± s.d.	99.0 ± 0.6	98.4 ± 0.8	99.1 ± 0.5	99.3 ± 0.7	98.6 ± 0.8	99.2 ± 0.3	98.9 ± 0.4	99.0 ± 0.6	98.6 ± 0.6	98.8 ± 0.5
Concentration range (M)	5×10^{-6} -1×10^{-4}	5×10^{-6} -7×10^{-5}	5×10^{-6} -5×10^{-5}	5×10^{-6} -1×10^{-4}	5×10^{-6} -5×10^{-5}	5×10^{-6}–1×10^{-4}	5×10^{-6}–1×10^{-4}	5×10^{-6}–1×10^{-4}	5×10^{-6}–1×10^{-4}	5×10^{-6}–1×10^{-4}
Calibration curve	A = 15900 C + 0.014	A = 24000 C – 0.039	A = 25000 C – 0.012	A = 25887 C – 0.04	A = 26173 C + 0.02	AUC = 1.262 × 10^{10}C + 2842	AUC = 2.005 × 10^{10}C + 1578	AUC = 1.6285 × 10^{10}C + 6939	AUC = 2.21 × 10^{10}C – 7536	AUC = 3.53 × 10^{10}C + 159923
	$r = 0.9998$, $n = 9$	$r = 0.9997$, $n = 8$	$r = 0.9998$; $n = 9$	$r = 0.9997$; $n = 9$	$r = 0.9998$; $n = 9$	$r = 0.9999$, $n = 9$	$r = 0.9996$, $n = 9$	$r = 0.9999$, $n = 6$	$r = 0.9999$; $n = 8$	$r = 0.9999$; $n = 8$
Detection limit (M)	6.4×10^{-7}	1.1×10^{-6}	1.0×10^{-6}	1.5×10^{-6}	2.2×10^{-6}	3.3×10^{-7}	3.8×10^{-7}	1.8×10^{-6}	8.4×10^{-7}	2.4×10^{-6}
Quantitation limit (M)	1.8×10^{-6}	2.8×10^{-6}	4.3×10^{-6}	3.4×10^{-6}	4.1×10^{-6}	5.8×10^{-7}	1.2×10^{-6}	4.4×10^{-6}	1.3×10^{-6}	4.5×10^{-6}

tain a final concentration of 1×10^{-3} M. Each solution was protected from light by using amber glass material.

4.2.3. Work solutions

2.5 mL aliquot of each stock solution of NB and BNB was taken and then diluted to 25 mL with ethanol/0.1 M Britton–Robinson buffer solution (final proportion of 30/70). In the case of PNB, PMNB and PCNB 2.5 mL of stock solutions was diluted to 25 mL in acetonitrile/0.1 M Britton–Robinson buffer solution (final proportion of 20/80).

4.3. Apparatus

4.3.1. HPLC

Measurements were carried out by using a Waters assembly equipped with a model 600 Controller pump and a model 996 Photodiode Array Detector. The acquisition and treatment of data were made with the Millenium Software, version 2.1. As chromatographic column a Bondapak/Porasil C-18 column of 3.9 mm × 150 mm was used. As column guard a C18 Kromasil 100-5C-18 (150 mm × 4.6 mm) was employed. The injector was a 20 μL Rheodyne valve. UV detection was employed, and the column was kept at constant temperature using a Waters column heater cartridge model 600.

For NB a gradient system composed by methanol/water (20/80) for 1 min to reach methanol/water (80/20) in 5 min was employed (1.2 mL/min, 40 °C, $\lambda = 270$ nm, run time 18 min).

For BNB an isocratic elution composed by acetonitrile/0.05 M phosphate buffer, pH 4.5 (55:45), was selected (1.2 mL/min, 20 °C, $\lambda = 250$ nm, run time 10 min).

For PNB an isocratic elution composed by acetonitrile/water (60:40) was used (1 mL/min, 20 °C, $\lambda = 261$ nm, run time 10 min).

For PMNB a gradient system composed by acetonitrile/water (15/85) for 2 min (1.2 mL/min) to reach acetonitrile/water (65/35) in 1 min (1.2 mL/min, 30 °C, $\lambda = 263$ nm, run time 10 min) was employed.

For PCNB an isocratic elution composed by acetonitrile/water (65:35) was selected (1.0 mL/min, 20 °C, $\lambda = 256$ nm, run time 8 min).

4.3.2. Spectrophotometer

Spectrophotometric measurements were carried out with an UV–Vis spectrophotometer ATI Unicam model UV2, using 1 cm quartz cell and equipped with a Pentium computer with Vision 2.2 acquisition and treatment program.

4.3.3. Electrochemical measurement

All the studies were carried out in aprotic media (dimethylformamide containing 0.1 M tetrabutylammonium perchlorate) using an electrochemical BAS equipment Model 50 W. A hanging mercury drop electrode, a Calomel electrode and a platinum wire electrode were used as the working electrode, reference electrode, and auxiliary electrode, respectively. NB, BNB, PNB, PMNB and PCNB were employed at 1.0×10^{-3} M concentration.

4.4. Analytical procedure

4.4.1. Calibration curve preparation

UV spectroscopy. NB and BNB stock solutions were diluted in ethanol/0.1 M Britton–Robinson buffer, pH 3.0 for NB and pH 5.0 for BNB (final ratio of 30/70). In the case of PNB, PMNB and PCNB, stock solutions were diluted in acetonitrile/0.1 M Britton–Robinson buffer (final ratio of 20/80), adjusted to pH 6.0. Working solutions ranging between 1×10^{-6} and 1×10^{-4} M were prepared.

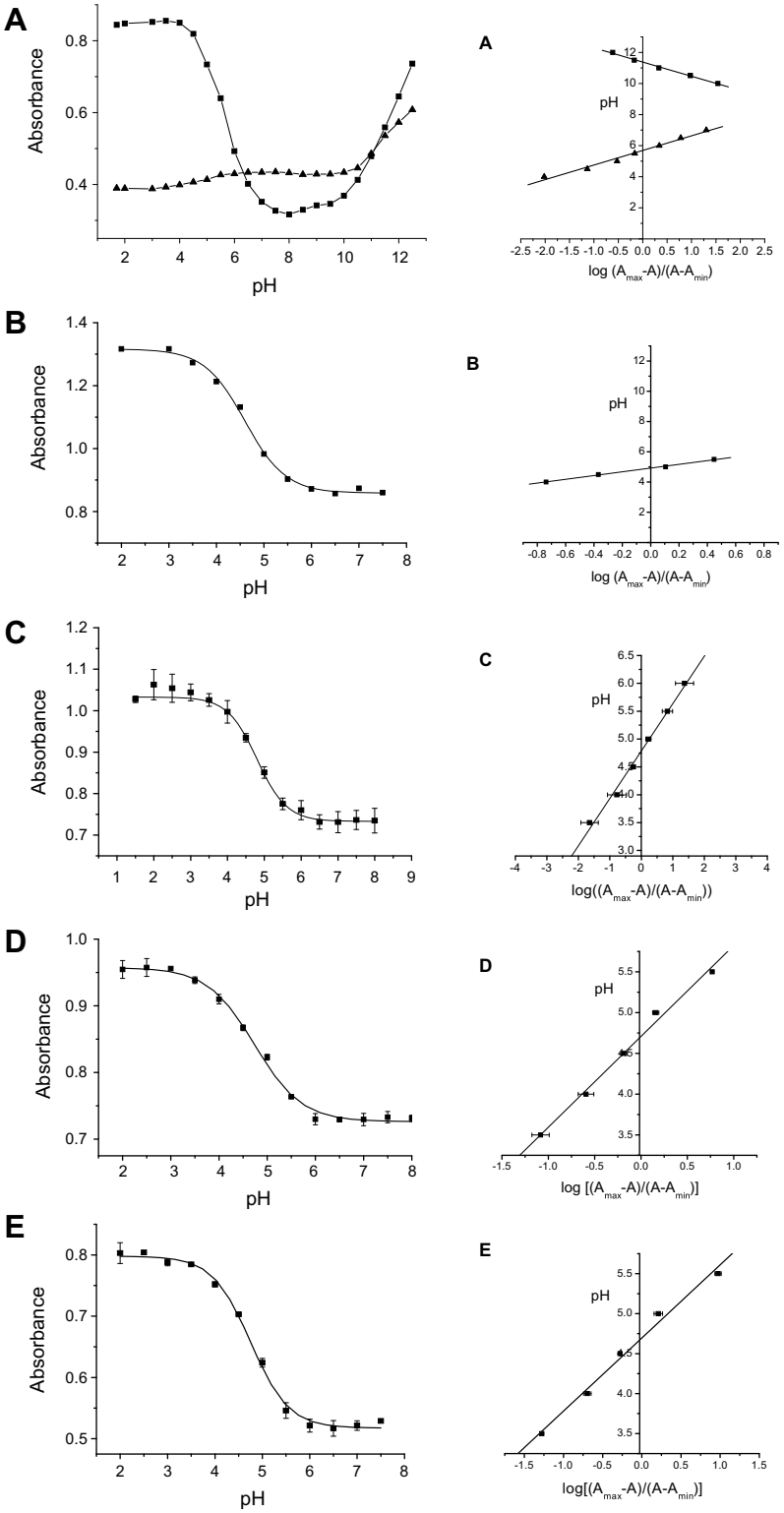

Figure 4. Absorbance–pH dependence for (A) NB at ■ 226 and ▲ 288 nm; (B) BNB at ■ 223; (C) PNB at ■ 230; (D) PMNB at ■ 230; and (E) PCNB at ■ 228. In every figure is inserted modified Hendersson–Hasselbach plots are inserted. (NB and BNB in ethanol/0.1 M Britton–Robinson buffer 30/70, PNB, PMNB and PCNB in acetonitrile/0.1 M Britton–Robinson buffer 20/80.)

HPLC. By diluting NB, BNB, PNB, PMNB and PCNB stock solutions with mobile phase, working solutions ranging between 1×10^{-6} and 1×10^{-3} M were prepared. The solutions were injected and chromatographed according to the working conditions previously given.

4.4.2. Selectivity studies[28]

Hydrolysis. 1 mL of stock solution of NB, BNB, PNB, PMNB and PCNB was disposed in a 10 mL-distillation flask, and was added (a) 5 mL 0.1 M HCl for acid hydrolysis or (b) 5 mL 0.1 M NaOH for basic hydrolysis. Then each solution was boiled for 3 h at reflux.

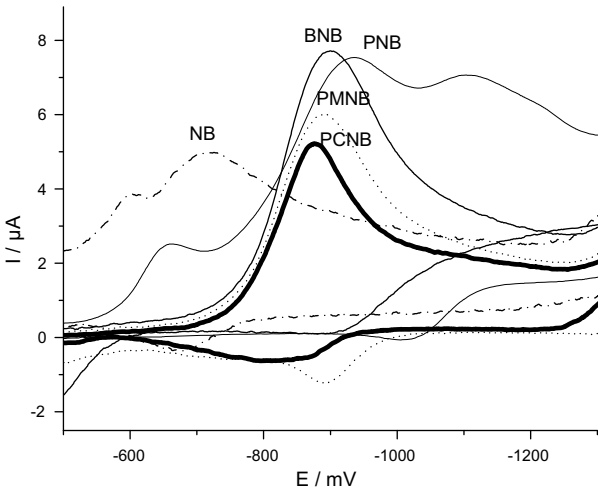

Figure 5. Cyclic voltammograms of 1.0×10^{-3}M solutions of NB, BNB, PNB, PMNB and PCNB in aprotic media at 1 V/s.

Photolysis. Separately 3 mL stock solution of NB, BNB, PNB, PMNB and PCNB in UV-cells was put on a black box and then irradiated with UV light (UV Black-Ray long wave ultraviolet lamp, UVP model B 100 AP, 50 Hz, 2.0 A, with a 100 W Par 38 Mercury lamp equipped with a 366 nm filter) at a distance of 15 cm for 8 h (1.2×10^{19} quanta/s, determined by using the potassium ferrioxalate chemical actinometer).[29]

Appropriate volumes of each obtained solution from the degradation trials were taken and completed to a final volume with mobile phase. Each sample was analyzed by duplicate.

Recovery studies. In order to evaluate the selectivity before possible interferences owing to common tablet excipients, mixtures of excipients (cornstarch, magnesium stearate, lactose, sodium lauryl sulfate and microcrystalline cellulose) were added to each compound for recovery studies, according to typical manufacturer's batch formulas for tablets. Each sample was suspended in the appropriate mobile phase volume and filtered prior to assay.

4.4.3. HPLC system suitability test

1.0×10^{-3} M of NB, BNB, PNB, PMNB and PCNB solutions was combined with their corresponding degradation products, synthesis precursors and KNO_3 as no retaining compound. The effects of different proportions (40%, 50%, 60%, 70% and 80%) of acetonitrile/methanol (for NB and BNB) and of acetonitrile/water (for PNB, PMNB and PCNB) were tested to establish the best analytical conditions by the determination of the capacity factor (k'), resolution (R), relative retention (α) and tailing factor (T).

4.5. Ionization constants determination (pK_a)

For the pK_a value determination, different wavelengths for each compound were assayed: 226 and 288 nm UV bands for NB; 223 nm for BNB; 230 nm for PNB, 230 nm for PMNB; and 228 nm for PCNB were used. The pH solution was changed each 0.5 unit, and next to the pK_a zone it was adjusted each 0.25 pH unit. 5×10^{-5} M solutions of each compound (NB and BNB in ethanol/0.1 M Britton–Robinson buffer 30/70, PNB, PMNB and PCNB in acetonitrile/0.1 M Britton–Robinson buffer 20/80) were employed separately for all the pH ranges, and the temperature was kept constant at 25 °C. pK_a values were obtained by plotting pH vs log $[(A_{max} - A)/(A - A_{min})]$ from modified Henderson–Hasselbach equation.[30]

4.6. Partition coefficients determination (log*P*)

The lipophilicity of each studied compound was measured by a HPLC method recommended by the Organization for Economic Cooperation and Development.[31] The analytical process was carried out with a Waters chromatograph system as described above. The column used for elution was a Bondapak/Porasil C-18 (3.9 mm × 150 mm). The mobile phase used was water/methanol (25/75) (v/v), and the flow rate was 1.0 mL/min. The water–methanol system (under isocratic condition) was used, and the retention time of NB, BNB, PNB, PMNB and PCNB was then determined. Since the log P value of a compound is related to its retention time in HPLC analysis, these values were calculated utilizing the following expressions:

$$\log P = \log k + C,$$

$$\log P = \log \frac{t_R - t_0}{t_0} + C,$$

where t_R is the retention time of the test compound, t_0 is the dead time, C is the intercept on the y-axis (log P) and k represents the capacity factor. Quantification is not required, and the determination of retention times is necessary only. A calibration curve was established by plotting the t_{Rs} to log$P$ values of six chemicals (benzoic acid, benzophenone, thymol, diphenylamine, naphthalene and phenanthrene) and the calculation of log$P$ values of NB, BNB, PNB, PMNB and PCNB was determined by their t_{Rs}.

4.7. Effective permeability constants determination (*Pe*)

The parallel artificial membrane permeability assay (PAMPA) was carried out at three different concentrations for each assayed compound: 5.0×10^{-6}, 7.0×10^{-6}, 1.0×10^{-5} M for NB; 5.0×10^{-5}, 7.0×10^{-5}, 1.0×10^{-4} M for BNB; and 5.0×10^{-5}, 3.5×10^{-5}, 2.0×10^{-5} M for PCNB, dissolved in 0.01 M phosphate buffer, pH 7.4, containing 1% of DMSO. L-α-phosphatidylcholine in *N*-dodecane was employed as membrane. Each solution was incubated at 25 °C for 4 and 16 h. Thiopental (40 μg/mL) and clidinium bromide (800 μg/mL) were used as positive and negative controls, respectively. The quantification was carried out using the developed spectrophotometric and chromatographic methods. Effective permeability values were calculated using the following expression:[32,33]

$$Pe = \frac{-218.3}{t} \log \left[1 - \frac{2C_{a(t)}}{C_{d(t0)}}\right] \times 10^{-6} \text{ cm/s},$$

where t is the incubation time, $C_{a(t)}$ is the concentration in the acceptor cell at the time of incubation and $C_{d(t0)}$ is the concentration in donor cell at zero time.

4.8. Assays on cultures

4.8.1. Cell culture and media

Trypanosoma cruzi (Strain MF) epimastigotes were grown at 28 °C in monophasic Diamond's culture media supplemented with 4 μM of hemin and 4% inactivated bovine calf serum. NB, BNB, PNB, PMNB and PCNB were added from stock solutions (10 mM in DMSO) to the culture obtaining final concentrations ranging from 1 to 100 μM.[34]

4.8.2. Growth inhibition assay

The effect of the studied compounds on growth constant (k_c) of *T. cruzi* epimastigotes (3×10^6 cell/mL) was determined by nephelometry, following the cell culture growth for 7 days. Nephelometry readings were directly proportional to the concentration of parasites. The k_c values were obtained from the epimastigotes

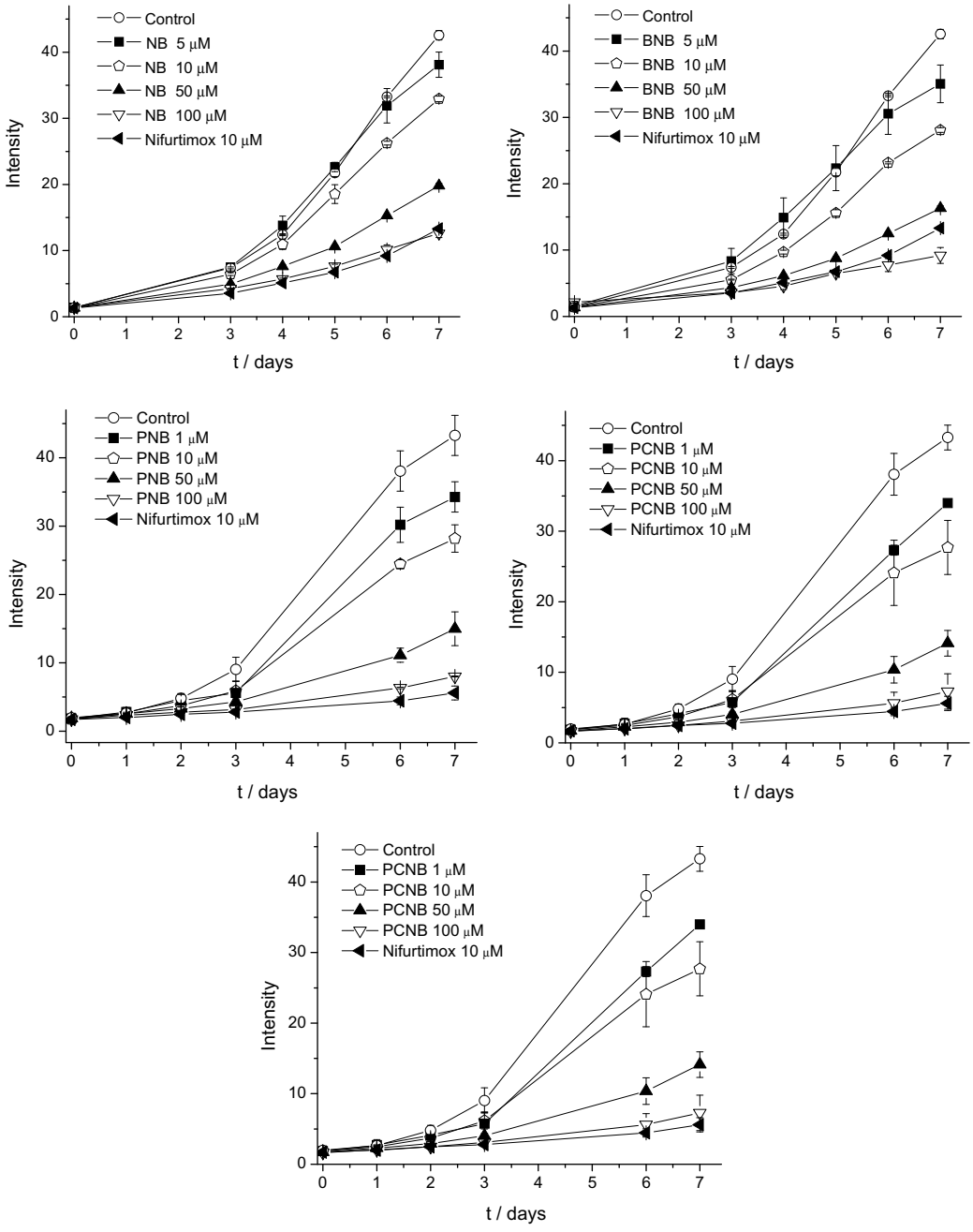

Figure 6. Effect of NB, BNB, PNB, PMNB and PCNB at different concentrations on *T. cruzi*'s growth in epimastigote form. Comparison with Nifurtimox.

exponential growth phase in the presence of the benzimidazoles (regression coefficient >0.97, $P < 0.05$). The IC_{kc50} is defined as the concentration of drug needed to diminish the k_c in 50% with respect to the control. In the assay conditions, no toxic effect of DMSO on *T. cruzi* epimastigotes was observed.

Table 2
o-Nitrophenylbenzimidazole derivatives characterization results by UV and HPLC developed methods

Compound	pK_a	log P	*Pe* (cm/s)	IC_{50} (μM)	$-Ep_c$ (mV)
NB	5.69 ± 0.06 11.38 ± 0.03	2.2	0.8×10^{-5} 1.2×10^{-5}	117	715
BNB	4.90 ± 0.02	3.1	Retained by membrane	94	899
PNB	4.79 ± 0.04	2.8	–	80	936, 1110
PMNB	4.71 ± 0.04	3.2	–	64	700
PCNB	4.69 ± 0.04	3.6	Retained by membrane	78	650

4.8.3. Oxygen uptake measurements

Trypanosoma cruzi epimastigotes were harvested by centrifugation (10 min at 5000 rpm), washed with 0.05 M sodium phosphate buffer, pH 7.4, containing 0.107 M NaCl and resuspended in the same buffer solution to a final concentration equivalent to 1 mg protein/mL (80×10^6 parasite cells correspond to 1 mg of protein or 12 mg of fresh weight). Oxygen uptake was determined by polarography with a Clark 5331 electrode (Yellow Springs Instruments) in a 53 YSI model (Simpson Electric Co).[35] The experiments were carried out at 28 °C using a 2 mL chamber as previously described.[10]

4.9. Statistic analysis

Comparison between different techniques, as well as the comparison with standard deviations, was carried out by means of the Student's *t* test, and using significance limits between 95% and 99% of confidence.[36,37]

Acknowledgement

Authors are grateful for the support of FONDECYT Grant No. 1061144.

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