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ORIGINAL PAPER

Identification of *N*-benzylacetamide as a major component of human plasmatic metabolic profiling of benznidazole

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Abstract Chagas disease is an endemic infection in Latin America with a high health impact. Caused by the parasite *Trypanosoma cruzi*, it has expanded to non-endemic regions such as North America and European countries via immigration of infected people. This infectious disease has been rising in the ranking of international health priorities due to the growing migration flows from endemic to non-endemic areas. Benznidazole (BZN), a nitroheterocyclic drug, is one of the two trypanocidal drugs currently in clinical use, associated with significant adverse drug reactions (ADRs). Mammalian metabolism of BNZ has been poorly studied, including the potential role of metabolites on both toxicity and anti-parasitic activity. High-resolution UPLC/MS/MS was used to analyze three plasma samples obtained from pediatric patients under BNZ treatment in steady state. Spectroscopic and structural criteria were applied to identify BNZ and accompanying substances from chromatographic signals. From all detected species, two can be undoubtedly associated with the BNZ and *N*-benzylacetamide molecules, the second one being a fragment of the parent drug (BZN). From the obtained results, two hypotheses could be formulated. The first one is to relate the presence of *N*-benzyl acetamide with the hepatic metabolism of BNZ. The second hypothesis has to do with the possible trypanocidal activity of this metabolite, as well as its role in the development of side effects, associated with the pharmacotherapy. Complementary studies should be carried out to determine the possible association of this metabolite with the BNZ treatment stages, patient's clinical features, ADRs, and trypanocidal effectiveness.

Keywords Nitrocompounds · Benznidazole · *N*-benzylacetamide · Chagas disease · Metabolite · UPLC/MS/MS · Plasma · Pediatric pharmacology

1 Introduction

Nowadays, approximately 8 million people in Latin America are infected with Chagas disease, caused by the parasite, *Trypanosoma cruzi* (Organización Panamericana de la Salud 2006). Also, Chagas disease has expanded to non-endemic regions such as North America and European countries via immigration of infected individuals (Schmunis 2007) and has been rising in the ranking of international health priorities due to the growing migration flows from endemic to non-endemic areas (Di Girolamo et al. 2011). The nitroheterocyclic drug benznidazole (BNZ) is one of the only two trypanocidal drugs currently in clinical use (Garcia-Bournissen et al. 2009; Richle 1973). *N*-benzyl-2-nitroimidazole acetamide is the chemical name of BNZ, wherein the nitroimidazole group is the pharmacophore for trypanocidal action and the benzyl group is the chromophore for most available analytical methods.

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Knowledge about metabolism of BNZ in humans is very limited, and the available information from animal studies is also scarce (Moreno et al. 1982; Richle and Raaflaub 1980; Workman et al. 1984, 1986; Lee and Workman 1986; Lee et al. 1987; Walton and Workman 1987). Regarding the pharmacology of BNZ from a study in healthy volunteers, the pharmacokinetic of this drug follows a one-compartment model (Lau et al. 1992; Raaflaub and Ziegler 1979; Raaflaub 1980). The peak plasma concentration was observed 3–4 h post dose, the estimated elimination half-life ($t_{1/2}$) was 12 h, and the apparent volume of distribution (V_d) was 0.56 L/kg (Raaflaub and Ziegler 1979). In a study of multiple doses of BNZ in adult chagasic patients (Raaflaub 1980), doses of 7 mg/kg produced overall plasma concentrations similar to those estimated from the pharmacokinetic parameters in the previous study in healthy volunteers (Raaflaub and Ziegler 1979). BNZ is apparently metabolized by the liver, the amount of unchanged drug excreted by the kidney being lower than 20 %.

The use of this drug is problematic, as it can cause significant side effects (Castro et al. 2006). The efficacy of BNZ is inversely related with the age of the patient (Streiger et al. 2004), with adverse drug reactions (ADRs) more frequently observed in adults (Cançado 2002). In pediatric patients, most ADRs are observed in children over the age of 7 and rarely in infants (Altcheh et al. 2011).

To sum up, human metabolism has been poorly studied, with the potential role of its metabolites in the observed toxicity as well as in the antiparasitic activity not described so far.

In this report, we describe the detection and identification of a plasmatic substance derived from pediatric patients treated with BNZ in a therapeutic protocol for Chagas disease. The detection of this substance, although not identified, was previously reported by our work group (Marson et al. 2013).

A brief discussion of its potential origin and possible role in the pharmacology and toxicology of the parent drug is now carried out.

2 Materials and methods

2.1 Patient selection and ethics statement

Forty children (2–12 years old) were enrolled between April 2008 and November 2010 into a prospective pediatric population pharmacokinetic study of BNZ. The enrollment was made in the Parasitology and Chagas Service, of the Buenos Aires Childreńs Hospital "R. Gutierrez", Argentina. The written informed consent was provided by the parents or guardians on behalf of all child participants, as well as the patients able to understand the procedure. The clinical protocol was approved by Ethics and Research Review Committee, Buenos Aires Childreńs Hospital "Ricardo Gutierrez", and the Argentine National Drug and Food Administration (ANMAT), Ministry of Health, Argentina. This clinical study was registered in clinicaltrials.gov (#NCT00699387). The patient features are described in Table 1.

The treatment and follow-up protocol were in agreement with current pediatric treatment guidelines (WHO 2002). BNZ (Radanil®, Roche, São Paulo, Brazil) 100 mg tablets, dose 5–8 mg/kg/d bid p.o. for 60 days (Altcheh et al. 2011). Tablets were fractionated by a hospital pharmacist, and written indications for administration were provided. Medication was provided in monthly batches, and compliance was assessed by tablet counting at each visit.

2.2 Patient plasma samples and pretreatment

Plasma samples of all patients were obtained from venous blood and BNZ was measured, most of the patients, in three different moments of treatment: at starting, in steady state and after finishing treatment, with previous reported HPLC/UV method (Marson et al. 2013). In these measures, the samples of patients in steady state showed a particular HPLC chromatographic pattern, compared with samples of patients at the beginning of the treatment. This chromatographic pattern involve some peaks clearly associated with those already founded for BNZ treatment, but could not be structurally identified by HPLC/UV instrumentation (Marson et al. 2013). From the above-described group, three representative samples were selected according to the chromatographic profile and the resolution of our peaks of interest (Table 1) for further identification analysis, using MS/MS detection.

2.3 UPLC/MS/MS instrumentation

Acetonitrile extracts were obtained with previously reported method (Marson et al. 2013). These extracts were analyzed using a series liquid chromatograph 1200 RRLC

Table 1 General features of three patients studied

Patient	1	2	3
Age (year/month)	2/9	5/0	9/10
Weight (kg)	11.9	20.0	27.0
BNZ administered per day (mg)	100	100	200
BNZ dose (mg/kg)	8.4	5.0	7.4
Last doses before sampling (h)	6.1	8.0	7.2
Treatment length before sampling (days)	14	8	8
BNZ HPLC/UV dosage (mg/L)	1.08	2.09	7.13

(Agilent) with a DAD detector in serial with a Bruker micrOTOF-QII electrospray source (ESI) high-resolution mass spectrometer. Samples (20 µl) were separated on a Phenomenex Luna C18 3u 100A (100 × 2 mm) and were eluted in an isocratic mode 70 % water (0.1 % formic acid): 30 % acetonitrile at a flow rate of 0.2 mL/min. Analytes were detected using a diode array and their *m/z* values were determined by positive electrospray ionization (ESI). Positive ESI-tandem mass spectrometry (MS) was performed in automatic mode. MS was carried out with a drying gas temperature of 200 °C, a drying gas flow of 8.0 L/min, a nebulizer gas pressure of 4 bar, and a capillary voltage of 150.0 V in full-scan mode in the *m/z* range of 50–900.

3 Results

All three samples showed similar features in the HPLC/UV (Fig. 1) and UPLC/MS/MS (Fig. 2) chromatograms, with two signals, the first peak (peak A) appearing at 5.3–6.1 min (with a maximum at 5.8 min) and a late eluting peak (peak B) at 9.9–10.8 min (with a maximum at 10.4 min). Both peaks were not symmetrical and showed a pre-shoulder. Peak A showed a clear resolution with two maximum at 5.4 and 5.8 min.

The MS spectrum of peak A (Fig. 3) was composed of a parent molecular ion $[M + H]^+$ that appeared at 261 *m/z* and a minor signal of molecular ion $[M+Na]^+$ that appeared at 283 *m/z*. The tandem mass spectrum of the parent molecular ion $[M+H]^+$ (261 *m/z*), also showed in Fig. 3, consisted on four principal fragmentation products of 214, 148, 107, and 91 *m/z* for all of the three samples. The MS/MS spectrum showed equivalent profile throughout the entire chromatographic signal between 5.3 and 5.9 min, including the principal chromatographic peak and its shoulder. The analysis of the exact mass of the principal ion indicates that this feature is compatible with the molecular formula $C_{12}H_{13}N_4O_3$, as the $[BNZ+H^+]$ ion, and the analysis of the exact mass of secondary ion is only compatible with the molecular formula $C_{12}H_{12}N_4O_3Na$, as the $[BNZ+Na^+]$ ion.

The MS spectrum of the chromatographic peak at 10.3 min is showed in Fig. 4. The tandem mass spectra of the parent molecular ion $[M+H]^+$ that appeared at 150 *m/z* is also showed in Fig. 4, is compatible in exact mass with $C_9H_{12}NO$ molecular structure, and can be assigned to the *N*-benzylacetamide molecule (BAA). In tandem spectrum of molecular ion at 150 *m/z*, there are three principal fragmentation products at 135, 120, and 107 *m/z* for all the samples. This ion series is compatible in exact masses with C_8H_9NO, $C_8H_{10}N$ and C_7H_9N molecular structures, respectively. These structures can be assigned to *N*-benzyl formamide, *N*-methylidene(phenyl)methanaminium, and benzyl ammonium molecules, all structurally related to the BAA. The fragment pattern assignations for tandem mass spectrum of peak at 150 *m/z* is showed in Table 2. The MS/MS spectra showed equivalent features

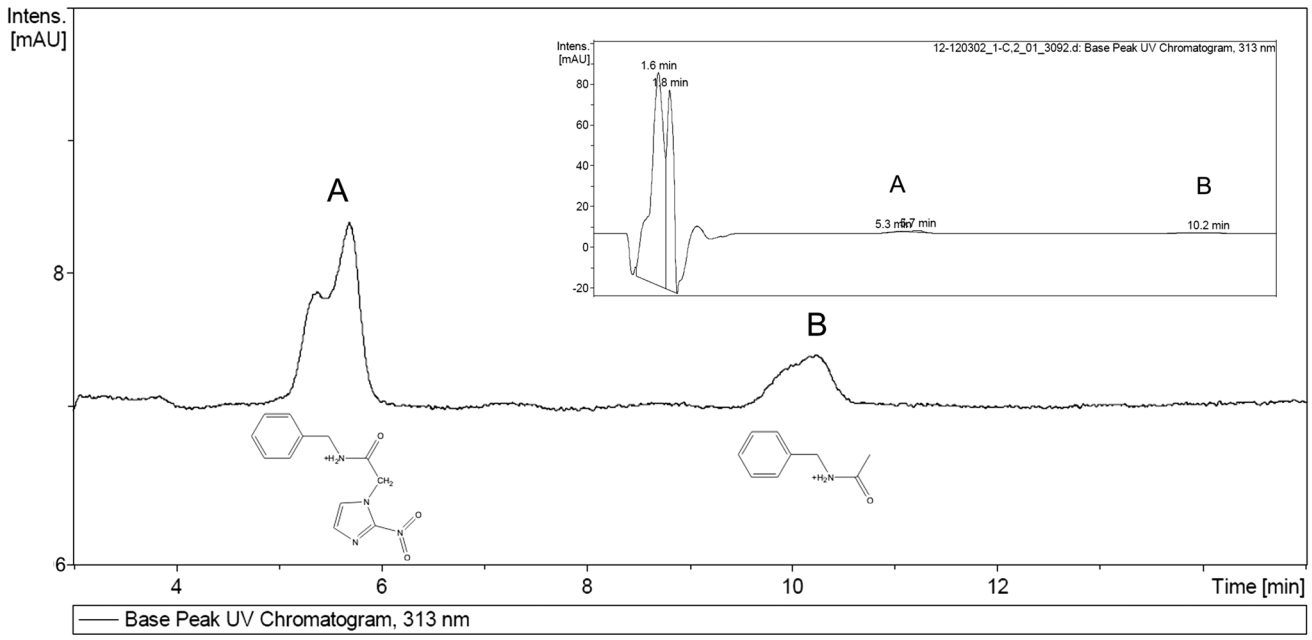


Fig. 1 Representative UV at 313 nm trace of plasma acetonitrile extract for elution between 3 and 15 min. Detail corresponding to the UV at 313 nm chromatogram for total elution time. Non resolved peaks eluting at 1–3 min are unbound material. Peaks specifically associated with benznidazole are *marked* (peaks A and B)

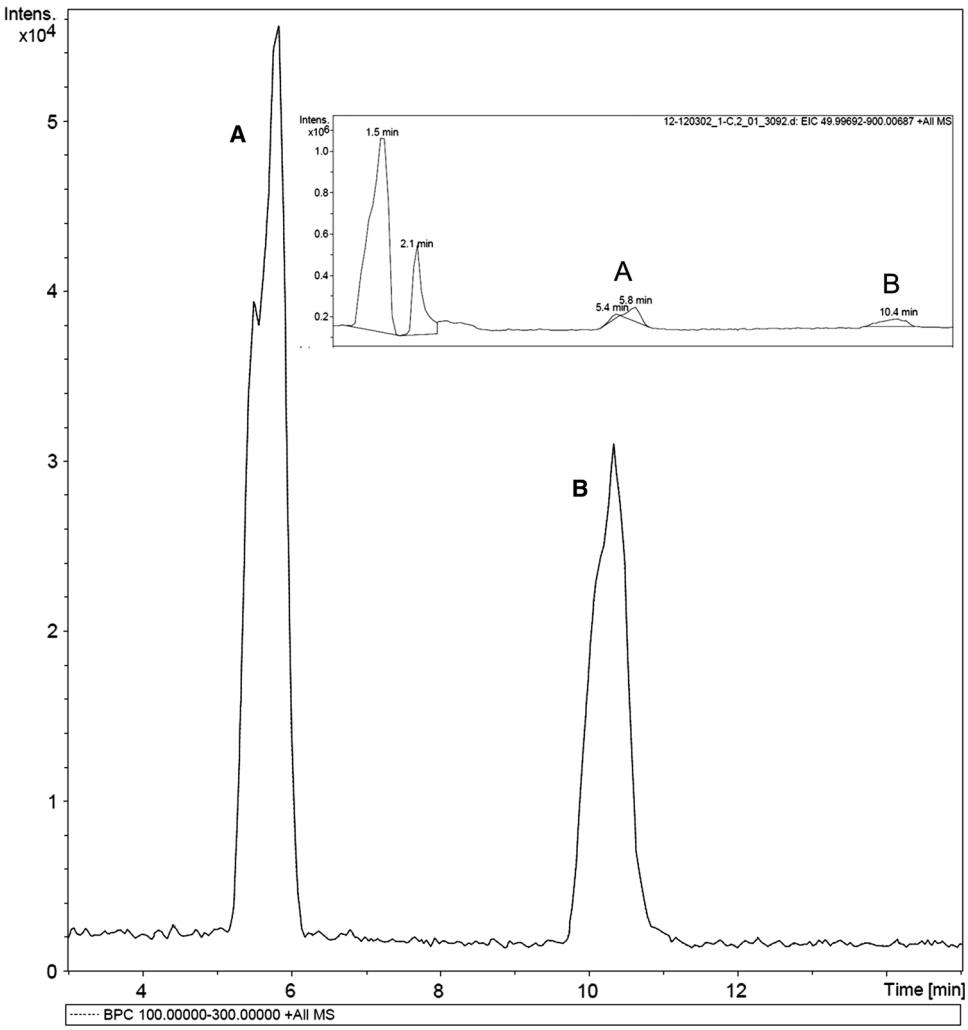


Fig. 2 Representative mass spectrometry chromatogram of plasma acetonitrile extract in the range 100–300 *m/z* for elution between 3 and 15 min. Detail corresponding to the mass spectrometry chromatogram in the range 50–900 *m/z* for total elution time. Non resolved peaks eluting at 1–3 min are unbound material, predominantly salts and acetonitrile. Peaks specifically associated with benznidazole are *marked* (peaks A and B)

through the entire chromatographic signal between 9.9 and 10.8 min.

4 Discussion

Using HPLC/UV, satellite chromatographic peaks associated with BNZ detection were previously described in clinical samples from pediatric chagasic patients (Marson et al. 2013).

In the present work, using an analog chromatographic system but with specific detection (MS/MS), the two species detected in the studied samples can be undoubtedly assigned to BNZ and BAA.

On the one hand, BNZ was detected, identified, and quantified in the same samples by an independent method using HPLC/UV (Marson et al. 2013). On the other hand, in mass spectra the features of the principal parent molecular ion $[M+H]^+$ and the secondary parent molecular ion $[M+Na]^+$ appeared at 261 and 283 *m/z*, both matching completely with BNZ structure (peak A).

Peak B, presents a tandem fragmentation pattern compatible with ions series described in the Table 2. Successive cleavage of alkyl structure as well as compatibility of exact masses of spectra features indicates without doubt the association of this peak with BAA molecule. Although there are no records of LC/MS for BAA, analogous profiles to our results were previously described using direct MS (Gilbert et al. 1975).

For quantitative purpose, the integrity of the sample must be ensured, using soft pre-treatment steps. In our protocol, no additional signals were detected using spiked serum samples with BNZ in quantitative calibration procedures (Marson et al. 2013). BNZ is a substance with known great stability. It is highly unlikely that the detected BAA could be the result of the degradation or the cleavage of the parent drug once the sample is obtained. It means that its detection could not be an artifact of the measurement.

Among the available data, human studies are scarce and obsolete and although it is highly possible that the BNZ hepatic metabolism depends on the P450 enzymatic family,

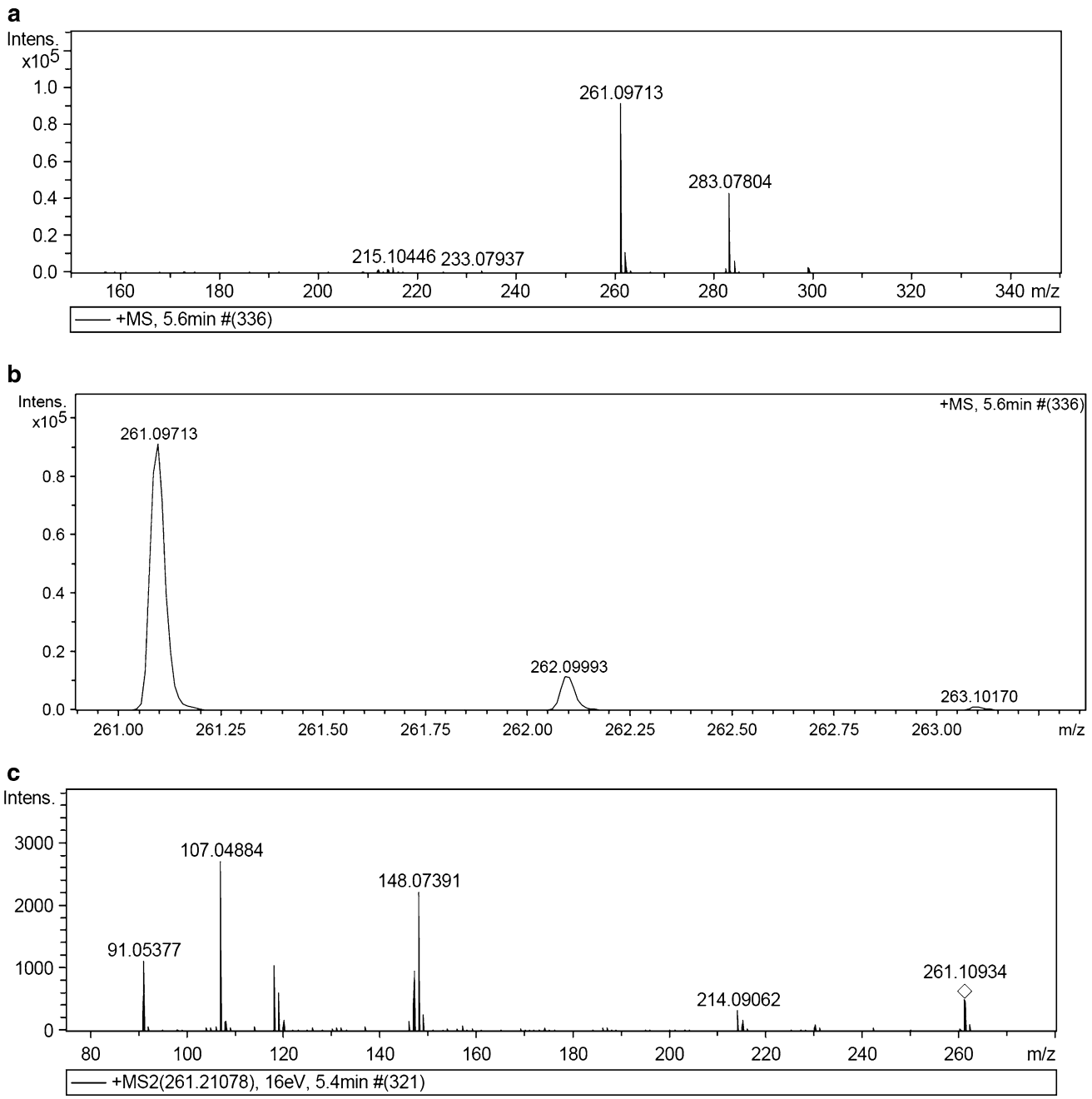


Fig. 3 **a** Positive ESI-MS for peak A (BNZ) of plasma ethyl acetate extract chromatogram. **b** Detail of the mass spectra of principal molecular ion $[M+H]^+$ at 261 *m/z* and **c** Tandem MS analysis of principal molecular ion from peak A (BNZ) shown in plasma ethyl acetate extract chromatogram

the apparent effect of different population polymorphisms is not clear to date (Castro et al. 2006; Lee and Workman 1984).

In accordance with our findings, early works described that by analogy with other nitroimidazoles, ring cleavage of BNZ should occur during hepatic metabolization (Workman et al. 1984). Moreover, in a recent work, a total nitro-imidazole group deletion product (α-amino-BAA) was found as a result of BNZ metabolization following trypanosomal type I nitroreductase reduction (Hall and Wilkinson 2012). Although no detectable parasite plasma metabolite concentrations are expected during antichagasic treatment, the presence of this specie as a metabolic product in other context shows a biological stability of benzyl structure.

Other results indicate the production of 2-hydroxy imidazole and 2-amino imidazole, as reduction products (Workman et al. 1984; Schwartz and Hofheinz 1982).

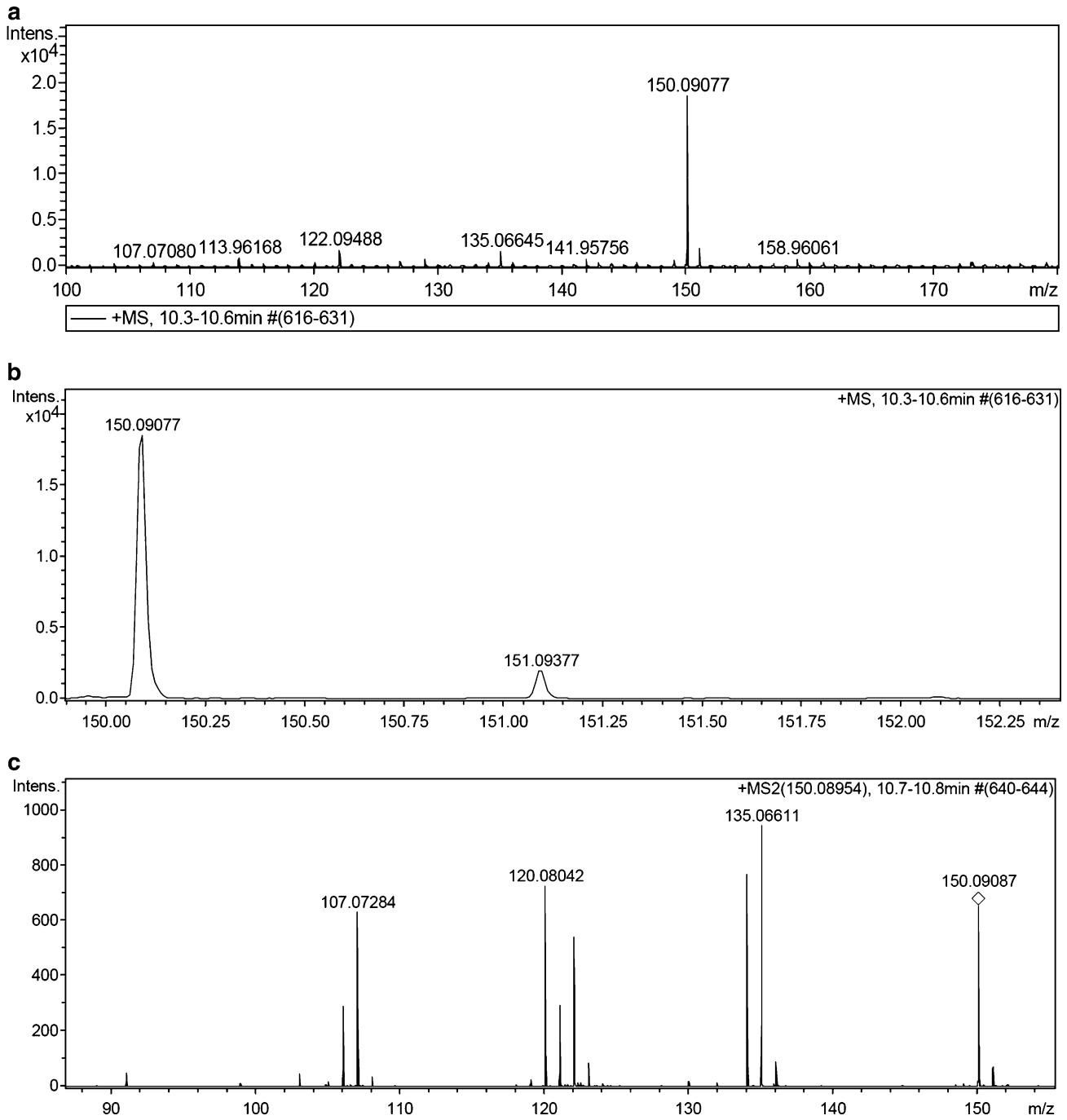


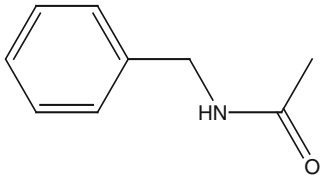
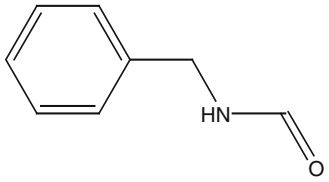
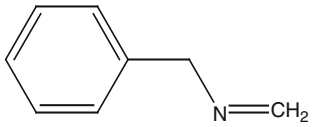
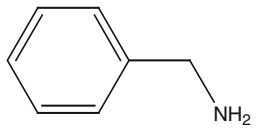
Fig. 4 **a** Positive ESI-MS for peak B (*N*-benzylacetamide) of plasma ethyl acetate extract chromatogram. **b** Detail of the mass spectra of principal molecular ion $[M+H]^+$ at 150 *m/z*, and **c** Tandem MS analysis of principal molecular ion from peak B (*N*-benzylacetamide) shown in plasma ethyl acetate extract chromatogram

Although both were generated also by the anaerobic reductive metabolism of BNZ in mammalian tissues (Walton and Workman 1987) and detected as products of its chemical reduction (Panicucci and McClelland 1989), no evidence of the amine or hydroxy BNZ derivatives was detected in our samples.

Our results, in accordance with most of the available data, indicate that it is highly probable that BAA derives from a series of metabolic reactions from BNZ and is a stable metabolic product.

The current hypotheses which attempt to explain toxicity of nitro compounds on parasites (trypanocidal

Table 2 Fragment pattern assignations for tandem MS of peak at 150 *m/z*

$[C_9H_{11}NO + H]^+$ Exact Mass: 150.09134 Mol. Wt.: 150.19713 m/e: 150.09189 (100.0%), 151.09524 (10.0%) C, 71.97; H, 8.05; N, 9.33; O, 10.65	 *N*-Benzyl-acetamide
$[C_8H_8NO+ H]^+$ Exact Mass: 135.06787 Mol. Wt.: 135.16261 m/e: 135.06841 (100.0%), 136.07177 (8.9%) C, 71.09; H, 6.71; N, 10.36; O, 11.84	 *N*-Benzyl-formamide
$[C_8H_9N+ H]^+$ Exact Mass: 120.08078 Mol. Wt.: 120.17115 m/e: 120.08132 (100.0%), 121.08468 (8.9%) C, 79.96; H, 8.39; N, 11.66	 *N*-methylidene(phenyl)methanamminium
$[C_7H_8N + H]^+$ Exact Mass: 107.07295 Mol. Wt.: 107.15251 m/e: 107.07350 (100.0%), 108.07685 (7.8%) C, 78.46; H, 8.47; N, 13.07	 Benzyl-ammonium

activity) and mammals (ADRs) require enzymatic reduction of nitro group with generation of reactive intermediates. Nitroheterocyclic compounds generally act as pro-drugs and must undergo activation to mediate their cytotoxic effects (Castro et al. 2006). Moreover, it has been suggested that BNZ serum concentrations do not appear to be related to the appearance of serious ADRs in adult chagasic patients (Pinazo et al. 2013). This fact strongly suggests a role of circulating metabolites in BNZ toxicity at therapeutic doses.

It would be possible to assign a significant role of circulating BAA in a similar concentrations range to BNZ on trypanocidal activity and/or mammalian toxic effects commonly observed in pediatric patients under BNZ treatment. In this sense, we selected steady-state plasma samples for MS/MS investigation because these clearly showed complex chromatographic patterns (HPLC/UV) in contrast with the plasma samples at the beginning of the treatment.

In relation to BAA there are only ancient records about its toxicity in mouse, which indicate effects on animal behavior and narcotic effects at doses near to the LD_{50} (550 mg/kg) (Aurousseau 1960).

To sum up, the age of the patients and the defined context of our pharmacokinetic study, and the limited number of samples used for ULPC/MS-MS analysis limit the validity and scope of the conclusions. Thus, a generalization of these findings is not recommendable. However, it is important to remark two main aspects of our findings: circulating levels of BNZ are lower in children than in adults (Altcheh et al. 2014) and the plasmatic detection of BAA as a possible metabolite, which is founded possibly in lower levels than BNZ.

In this sense, some noticeable differences in BNZ human pharmacology and toxicology for adult and children have been described (Cançado 2002; Altcheh et al. 2014; Hines 2008; Kearns et al. 2003). However, these results could not explain the highest therapeutic efficacy in

younger patients, as well as the age-related increase in toxicity. The results obtained with pediatric patients suggest the necessity for further studies about the relationship between age and plasmatic metabolites. Moreover, if we assume that the founded pattern in these three patients is a common phenomenon, we count with another working hypothesis to explain the observed differences on BNZ pharmacology and/or toxicology between adults and children.

In conclusion, a strong evidence of BAA as human BNZ plasmatic metabolite on pediatric patients treated with BNZ was described. The estimated plasmatic concentration of this substance is compatible with a hypothesis associated with a relative importance of it on antiparasitic activity and/or human toxicity. Up to now, this is the first report of a possible metabolite of BNZ in human being. This is noteworthy, since BNZ is approved for use in humans for more than 40 years. This fact is representative of drugs used in a called neglected disease such as Chagas disease.

Complementary studies should be carried out to confirm the BAA molecule as regular metabolite of BNZ and to elucidate the relationship between this substance and the pattern related to BNZ treatment, patients' characteristics, clinical features, ADRs, and trypanocidal effectiveness.

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