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Development of a LC–MS/MS methodology for the monitoring of the antichagasic drug benznidazole in human urine

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ABSTRACT

Monitoring the drug benznidazole in biological fluids is a powerful tool for clinical diagnostic and pharmacological studies in chagasic patients. However, research in this concern needs to be done. The accurate quantitation of this drug in complex matrices represents a highly challenging task complicated by the absence of sensitive analytical methods. It follows that sample processing strategies, preparation/cleanup procedures, and chromatographic/ionization/detection parameters, were evaluated for method optimization. The summation of this work generated a rapid, selective, sensitive methodology based on reversed-phase chromatography–tandem mass spectrometry for the analysis of benznidazole in urine samples. To the best of our knowledge, this is a first report of a LC–MS/MS platform employed for this application. Matrix effect was determined; a 90% of signal suppression was observed. The limits of detection and quantification were 0.75 and 4.85 $\mu g\,L^{-1}$; respectively. The latter allowed the method's application to the detection of benznidazole in clinical studies and pharmacological monitoring analysis.

1. Introduction

Chagas disease (or American trypanosomiasis) is a deadly potentially disease caused by the *Trypanosoma cruzi* protozoan parasite. The geographical distribution of this disease is extensive, including mainly America, with endemic characteristics in South America [1]. At present, the number of people infected with Chagas disease worldwide is estimated to be about 10–12 million, causing 10,000 deaths per year due to complications [2]. The process of urbanization in Latin America and migratory population movements from endemic countries have led to the disease being diagnosed in non-endemic areas [3,4].

T. cruzi is a single-celled parasite transmitted primarily by blood-sucking insects, popularly called “kissing bugs”. *T. cruzi* enters the digestive tract of the insect when it bites a person or an infected mammal. The parasite is actively divided inside the insect and is transmitted through its feces which are deposited on the skin host. This transmission path, known as vectorial, was considered the most common in the American region.

On the other hand, the non-vector transfer pathways are related to (a) transplacental transmission (congenital), (b) blood transfusion, (c) organ transplant, (d) oral, by food intake with parasites, and (e) laboratory accidents [5].

In recent years the advances in the control of vectorial and transfusional transmission have converted the congenital transmission in the main source of transfer, especially in urban areas [1,6,7].

Nowadays, the only drugs existing for Chagas treatment are benznidazole (BNZ) and nifurtimox [8–10]. The first (N-benzyl-2-(2-nitroimidazole-1-yl) acetamide), $C_{12}H_{12}N_4O_3$, MW: 260.25 g mol^{-1} (Fig. 1); was obtained for veterinary purposes by Roche in 1974 and the second was developed by Bayer in 1960. Both commercially available pharmacotherapies are inadequate due to issues involving safety, efficacy, resistance, toxicity, difficulty of administration in impoverished conditions, and cost. Response to treatment depends greatly on the disease phase [11–14]; so early detection is not only important but critical for public health.

BNZ is the first pharmacotherapeutic option in Americas, its oral ingestion for acute stage treatment varies from 5 to 8 mg kg^{-1} day^{-1} p.o. for 60 days [5]. However, information related to its pharmacokinetic distribution in special populations, such as lactating, children, elderly, and pregnant women, is poor or nonexistent. In addition, the chemotherapy with BZN requires prolonged administration

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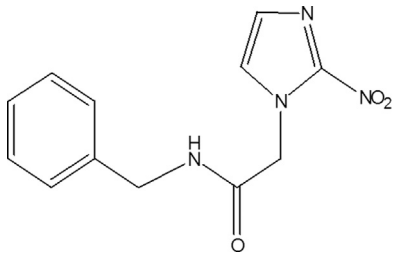

Fig. 1. Benznidazole structure.

(30–60 days) and has significant toxicity, especially in adults [15–17]. Moreover, the adverse drug reactions (ADRs) are the most important factor in therapeutic failure [18–20]. It follows the need for evaluation and monitoring of BNZ in biological fluids.

To date, the majority of studies focus on its therapeutic efficacy [21], pharmaceutical technology [22,23], preclinical pharmacokinetics evaluation [24] and metabolism study in animals [25,26]. There are only a few studies concerning to the quantification of this antichagasic drug by techniques such as electrochemistry [27,28], polarography [29], spectrophotometry [30,31] and liquid chromatography (LC) associated to UV detection [32–34]. However, it has been well documented the limitations of some of these analytical approaches to detect interferences and overcome some serious problems related to sample complexity. Moreover, only a few authors have developed LC–UV-based methodologies to quantify BNZ in plasma and animal urine for further implementation in human pharmacokinetic and health–safety studies [34–37]. A recent report, the unique one to the best of our knowledge, has been developed for applications in urine samples for studies in humans [37], with limited descriptions of analytical method performance.

Liquid chromatography coupled to mass spectrometric detection, in its various forms (LC–MS and LC–MS/MS), is an excellent alternative of analysis. The Ultra High Performance Liquid Chromatography (UHPLC) approach offers very important advantages associated finally with the time required for analysis. In many cases, it is necessary to use a cleaning/extraction/preconcentration procedure of the analytes in order to obtain the optimum conditions for the determination of them or to overcome the effects generated by the biological sample matrix. Following an exhaustive search in the scientific literature, it has been determined that a gold standard method employing a chromatographic approach coupled to mass spectrometry for the quantitative analysis of BNZ in biological samples has not been reported to date and it would be beneficial to the analytical community. Moreover, no studies applied to human samples with focus on clinical or therapeutic drug monitoring (TDM) have been reported. The analyte's determination in urine samples is important in order to obtain valuable information that could help to elucidate its pharmacokinetic behavior. Additionally, urine is a non-invasive sample, which is a valuable condition for TDM. In this study the optimization of a methodology for the extraction/chromatographic separation and mass spectrometric determination of BNZ in human urine samples is proposed. The optimized method provided a selective, sensitive, fast, and reliable strategy for the accurate detection of the antichagasic drug and, at the same time, it could be potentially used as a tool to generate relevant information for rational development of disease pharmacological therapy, today still scarce.

2. Experimental

2.1. Chemicals and reagents

BNZ analytical grade was donated by Hoffmann-La Roche Ltd. (Buenos Aires, Argentina). Water, methanol and acetonitrile Optima® LC–MS grade, were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Dichloromethane and trichloroacetic acid pro-analysis were purchased from Biopack (Buenos Aires, Argentina). Formic acid, 98%, was obtained from Fisher Scientific (Loughborough, UK). Ultrapure water (18 MΩ cm) was obtained from EASY pure (RF Barnstead, IA, USA). Organic standard solutions of BNZ were daily prepared by appropriate dilutions of a 100 mg L^{-1} aqueous stock solution. Quantification was achieved by preparing spiked urine samples with proper amounts of the analyte. The solutions were maintained at 4 °C protected from light. For sample pre-treatment and filtering prior to LC–MS/MS analysis, 13 mm nylon hydrophilic membranes of 0.8 and 0.22 μm diameter pore (Osmonics INC, USA) were used, respectively.

2.2. Instrumentation and conditions

Rotary evaporator Heidolph Laborota 4010 configured with ROTAVAP valve control equipment was used.

An Acquity™ Ultra High Performance LC system (Waters, Milford) equipped with autosampler injection and pump systems (Waters, Milford) was used. The autosampler vial tray was maintained at 15 °C. The needle was washed with proper mixtures of acetonitrile and water. The separation was performed by injecting a 10 μL sample onto an ACQUITY UPLC® BEH C_{18} (Waters, Milford, USA) analytical column with 2.1 mm internal diameter, 50 mm length, and 1.7 μm particle size. The binary mobile phases consisted of water with 0.1% (v/v) of formic acid (A) and acetonitrile with 0.1% (v/v) of formic acid (B) at a flow rate of 0.15 mL min^{-1}. The gradient was started at an initial composition of 90% A and 10% B, then 3-min linear gradient to 0% A, held for 0.3 min. A return to the initial conditions was accomplished by a 0.2-min gradient to 90% A, where it was held for 1.5 min. Thus, the total chromatographic run time was 5.0 min. The column was held at a temperature of 30 °C. Under these conditions, no sample contamination or sample to sample carryover was observed.

Mass spectrometry analyses were performed on a Quattro Premier™ XE Micromass MS Technologies triple quadrupole mass spectrometer with a ZSpray™ electrospray ionization source (Waters, Milford, USA). The source was operated in the positive (ESI+) mode at 350 °C with N_2 as the nebulizer and the source temperature was kept at 150 °C. The capillary voltage was maintained at 3.5 kV and the extractor voltage was set at 3.0 kV. Ultrapure nitrogen was used as desolvation gas with a flow of 200 L h^{-1}. Argon was used as the collision gas at a flow of 0.10 mL min^{-1}. Detection was performed in multiple reaction monitoring (MRM) mode of selected ions at the first (Q_1) and third quadrupole (Q_3). To choose the fragmentation patterns of m/z (Q_1)→m/z (Q_3) for the analyte in MRM mode, direct infusion (via syringe pump) into the MS of BNZ standard solution in methanol was performed and the product ion scan mass spectra were recorded. Quantification of BNZ was done by measuring the area under the peak using MassLynx Mass Spectrometry Software (Waters, Milford, USA).

2.3. Sample collection and preparation

Urine samples were collected in sterile specimen collection bottles during 24 h from a pediatric chagasic patient and an adult volunteer, both after a 5 mg kg^{-1} day^{-1} p.o. BNZ doses. The whole procedure was made anonymous and the patients were enrolled in a clinical study approved by both ethical and research review committees of Children's Hospital Ricardo Gutierrez, Buenos Aires city, Argentina. All patients were informed about the purposes and scope of the study, and signed appropriate consent forms. Data assessment was complete for all patients.

2.3.1. Sample treatment

After collection, samples were divided into three aliquots and stored at −21 °C until analysis. Thaw was performed prior the three fractions underwent the following treatments.

2.3.1.1. Filtering. A measured volume of urine was centrifuged for 10 min at 12,000 rpm. A 500 μL aliquot of the supernatant was diluted with water:acetonitrile up to 1 mL final volume, filtered through a 0.22 μm syringe filter and stored in amber vials suitable for LC–MS/MS analysis.

2.3.1.2. Dryness. 2.5 mL urine volume was mixed with 5.0 mL acetonitrile volume, stirred and centrifuged. Supernatant was removed and brought to dryness in a rotary evaporator. Then it was reconstituted in water:acetonitrile, filtered through a 0.22 μm syringe filter and stored in an amber vial suitable for analysis.

2.3.1.3. Liquid–liquid extraction. Two consecutive extractions with 1.25 mL of dichloromethane each was performed on a 2.5 mL urine volume. The organic phase was separated and brought to dryness in a rotary evaporator. Then it was reconstituted with water: acetonitrile and filtered prior LC–MS/MS analysis.

2.4. Evaluation of matrix effect

As it is known, one downside of ESI–MS/MS ionization/detection is that the ionization process is susceptible to matrix signal suppression or enhancement [38–40]. The liquid chromatography–mass spectrometry response obtained from a standard can differ significantly from matrix samples. In this work, after optimizing the compound selective parameters, the effect of the urine matrix was assessed by comparing the signal of BNZ in pure solvent to the signal in the diluted and non-diluted sample matrix. Calibration curves for BNZ in the spiked biological fluid and in the pure solvent were created. The percentage of the quotient of the slopes (b) in the spiked and solvent samples was used as an indicator of the extent of the matrix effect, which was calculated as $100-(b_{spiked}/b_{solvent}\times 100)$.

2.5. Assay performance

The calibration plots were measured under the optimal experimental conditions. Six levels of the calibration curve were determined (five technical replicates at each concentration level). The calibration equations were calculated by the least-squares linear regression method. Thus, linearity was evaluated from values closer to the limit of detection up to approximately 50,000 μg L^{-1}.

Intra-day repeatability, and inter-day reproducibility, spiked urine samples were analyzed. Thus, 6 blank samples, 5 replicate measurements at 0, 10, 100, 500, 1000, 5000, 10,000, 25,000, and 50,000 μg L^{-1} BNZ concentrations were prepared.

3. Results and discussion

3.1. Sample preparation optimization

Although the technology related to chromatographic separations and mass spectrometry techniques advances, sample clean-up is still one of the most important bottlenecks of the analytical process. Effective sample preparation is essential for achieving good analytical results because matrix related compounds may also co-extract and interfere in the analysis.

The selection of the sample clean-up conditions in the analysis of BNZ in biological material is a difficult task taking into account the nature of the analyte. Consequently, optimization of the clean-up steps paying special attention to the analytes recovery was carried out. The accuracy of the proposed method was evaluated by recovery experiments with spiking diluted sample solutions. Several approaches were considered, including filtering, dryness, and liquid–liquid extraction. The latter procedure demonstrated to be more proper in terms of the analyte's recovery, which resulted to be higher than 95%. Although, for clinical applications, recoveries equal or less than 70% with sufficient reproducibility may be appropriate. On the other hand, recoveries for sample filtering and dryness approaches ranged between 20% and 40%.

There has been little effort devoted to the study of drugs for the treatment of Chagas, a so-called “neglected disease”. It has been described that BNZ have hepatic metabolization with $<20\%$ of the drug excreted unchanged by the kidney. However, there are virtually no studies on the metabolism of BNZ in humans, and very little information is available from animal studies [24,41–43]. Furthermore, there are no complete data on drug excretion through the urine, partially because of the lack of available techniques, as the one developed in this work. Although the samples used in this experience for validation and application of the proposed technique might not be enough to obtain general conclusions regarding this aspect, the higher BNZ value obtained in the pediatric urine is compatible with the reported for lower plasma dosage and higher plasmatic clearance from children below seven years old under a pharmacotherapeutic treatment [44].

3.2. Optimization of MS parameters and MRM transitions

Preliminary experiments were conducted with the purpose of finding the best instrumental conditions that would allow identification of BNZ in urine samples at trace levels. BNZ standard solution (1 mg L^{-1}) in methanol was introduced into the MS system at a flow rate of 20 μL min^{-1} via a syringe pump. The ion-full scan in positive mode (mass spectra from m/z 50 to 300 were recorded) of BNZ indicated the presence of its pseudomolecular ion $[M+H]^+$ as the predominant specie, with m/z value of 261.1. Multiple reaction monitoring mode of the precursor–product ion transitions was optimized. For quantitative mass spectrometric detection, it is generally accepted that a minimum of three identification points are required to meet the identification performance criteria. In this work, monitoring of one precursor ion and three daughter ions “earned” 5.5 identification points (1 for the parent ion and 1.5 for each daughter ion) and, therefore, fulfilled the mentioned criteria giving the necessary specificity to identify a substance correctly. Specific charged fragments for the studied analyte are listed in Table 1. The optimization of the ions production and fragmentation conditions permitted the analyte's sensitive and selective detection. As a result, the area under the most sensitive transition: 261.1 > 107.3 was measured for quantification purposes.

3.3. Chromatographic procedure

To improve BNZ chromatographic retention, a reversed phase column was used and several mobile phases such as methanol, acetonitrile, and water were investigated. The results indicated

Table 1
Mass spectrometric conditions for the generation and fragmentation of BNZ.

Transitions (m/z)	Dwell (s)	Cone (V)	Collision (V)
261.1 > 148.2 261.1 > 107.3[a] 261.1 > 91.1	0.08	25	18

[a] Transition used for quantification.

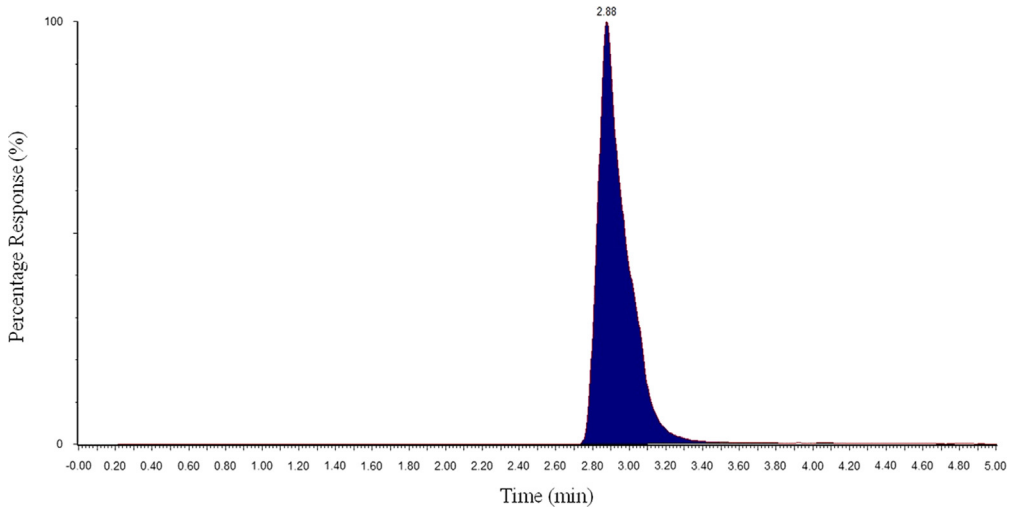

Fig. 2. Representative chromatogram of BNZ in spiked urine samples. Ionization and mass spectrometric conditions: electrospray ionization in positive mode associated to mass spectrometric detection in multiple reaction monitoring mode (experimental parameters as described in Section 2.2). Chromatographic approach: C_{18} column; mobile phase H_2O:ACN gradient mode containing 0.1% (v/v) formic acid; mobile phase flow rate: 0.15 mL min^{-1}; temperature: 30 °C; BNZ standard concentration: 100 μg L^{-1}; sample injection volume: 10 μL.

that a mixture of water/acetonitrile as gradient solvents were optimal to allow formation of a fine spray of small droplets in the ESI interface. To enhance the signal response, mobile phase additives such as acetic acid and formic acid were also studied. The type (acetic or formic acid) and concentration (from 0.05% to 4.0% (v/v)) of additive were evaluated. The use of formic acid led to improved peak shape and retention time compared to acetic acid. The use of either one gave improved retention time, peak shape, and sensitivity compared to no additive. As a result, a 0.1% (v/v) formic acid concentration provided the maximum response for the generation of the protonated $[M+H]^+$ BNZ ion, which was used for further MRM experiments. The effect of the mobile phase flow rate on the separation/retention of BNZ was evaluated using van Deemter plots. Ten microliters of the standard sample was injected onto the reversed phase system at varying flow rates, from 0.1 to 1.0 mL min^{-1} with isocratic separation. Thus, a flow rate of 0.15 mL min^{-1} gave the best results in terms of chromatographic conditions and ESI efficiency. Under the optimal mentioned conditions, the analyte was eluted at 2.88 min (Fig. 2) from the column within a 5.0 min total run cycle, which were shorter than the ones recently reported by other authors [33,34,36].

3.4. Evaluation of matrix effect

As mentioned in Section 2.4, after selecting the proper chromatographic conditions, the effect of the biological matrix under study (urine) was evaluated by comparing the signal of BNZ in pure solvent to the signal in the diluted and non-diluted sample matrix. Thus, calibration curves from spiked matrix and spiked pure solvent samples were created. Although analyte's liquid–liquid extraction was performed, BNZ response reduction of approximately 90% in both, diluted and non-diluted matrices, due to the urine interference was observed. As a consequence, its quantification was carried out following the standard addition method.

3.5. Analytical performance

A Certified Reference Material of the studied matrices with an informed value for BNZ does not exist. As a consequence, recovery of additions of known amounts of the analyte to a blank matrix was used. Thus, spiked urine samples were analyzed and recovery values better than 95.5% were achieved.

Table 2
Intra and inter-day variability for the LC–MS/MS analysis of benznidazole.

Concentration level (μg L^{-1})	Intra-day RSD (%)[a]	Inter-day RSD (%)[a]
10.0	3.9	7.0
100.0	3.7	5.8
500.0	3.2	6.5
1000.0	2.4	6.3
5000.0	2.8	6.2
10,000.0	2.1	6.2
25,000.0	2.0	6.3
50,000.0	2.1	6.0

[a] RSD (%)=Relative Standard Deviation.

As mentioned in Section 2.5, repeatability (intraday precision) and reproducibility (interday precision) were evaluated. The obtained results are summarized in Table 2. In summary and taking into account the matrix complexity, the reported values for the method assessment parameters could be considered highly satisfactory.

The limit of detection (LoD) and limit of quantification (LoQ) were calculated as the signal equivalent to respectively 3 times and 10 times the background chromatography noise under working conditions. Thus, the obtained LoD and LoQ concentration values were 0.75 ± 0.03 and 4.85 ± 0.05 μg L^{-1} respectively.

3.6. Sample analysis

Once the optimal conditions were established, the developed methodology was applied to the analysis of real urine samples. The mean BNZ concentration were 29.4 μg mL^{-1} and 22.6 μg mL^{-1} for the pediatric and the adult samples respectively.

4. Conclusions

A sensitive and selective analytical method for the chromatographic separation and determination of BNZ in urine samples based on the use of a reversed-phase liquid chromatography coupled to tandem mass spectrometry was developed and proposed for first time. The rapidness, selectivity, and sensitivity characteristics of the proposed methodology in the complex

matrix under study were demonstrated. Our studies showed that matrix effects should be carefully assessed when biological fluids are involved. Finally, methodology developed could be of great interest for monitoring BNZ variations in clinical and pharmacological studies.

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