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Development and validation of HPLC method with fluorometric detection for quantification of bisnaphthalimidopropyldiaminooctane in animal tissues following administration in polymeric nanoparticles

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ABSTRACT

A simple, sensitive and specific high-performance liquid chromatography method for the quantification of bisnaphthalimidopropyldiaminooctane (BNIPDaoct), a potent anti-*Leishmania* compound, incorporated into poly(D,L-lactide-co-glycolic acid) (PLGA) nanoparticles was developed and validated toward bioanalysis application. Biological tissue extracts were injected into a reversed-phase monolithic column coupled to a fluorimetric detector (λ_{exc} = 234 nm, λ_{em} = 394 nm), using isocratic elution with aqueous buffer (acetic acid/acetate 0.10 M, pH 4.5, 0.010 M octanesulfonic acid) and acetonitrile, 60:40 (v/v) at a flow rate of 1.5 mL min^{-1}. The run time was 6 min, with a BNIPDaoct retention time of 3.3 min.

Calibration curves were linear for BNIPDaoct concentrations ranging from 0.002 to 0.100 μM. Matrix effects were observed and calibration curves were performed using the different organ (spleen, liver, kidney, heart and lung) extracts. The method was found to be specific, accurate (97.3–106.8% of nominal values) and precise for intra-day (RSD < 1.9%) and inter-day assays (RSD < 7.2%) in all matrices. Stability studies showed that BNIPDaoct was stable in all matrices after standing for 24 h at room temperature (20 °C) or in the autosampler, and after three freeze–thaw cycles. Mean recoveries of BNIPDaoct spiked in mice organs were >88.4%. The LOD and LOQ for biological matrices were ≤0.8 and ≤1.8 nM, respectively, corresponding to values ≤4 and ≤9 nmol g^{-1} in mice organs. The method developed was successfully applied to biodistribution assessment following intravenous administration of BNIPDaoct in solution or incorporated in PLGA nanoparticles.

1. Introduction

Naphthalimides and bisnaphthalimides are cytotoxic DNA-intercalating compounds, with well-established activity against several cancers [1]. Bisnaphthalimidopropyl derivatives (BNIPs) linked to natural polyamines were designed and synthesized to exhibit good cytotoxicity against cancer cells and parasites [2–7]. In particular, bisnaphthalimidopropyldiaminooctane (BNIPDaoct, Fig. 1) was shown to exert promising activity against certain cancer cells (pancreas, breast and leukaemia) and *Leishmania infantum* protozoa, eliciting cell death by apoptosis with DNA damage [4,8,9]. However the lack of aqueous solubility and some toxic effects to normal cell at higher doses has made BNIPDaoct *in vivo* testing difficult and highly limited [4]. To overcome these problems, BNIPDaoct was incorporated into polymeric nanoparticles of poly(lactic-co-glycolic) acid (PLGA), a biodegradable and biocompatible polymer approved by Food Drug Administration for therapeutic applications. By applying such a drug delivery system one can reduce compound cytotoxic activity side effects, increase their aqueous solubility properties and alter compound pharmacokinetics profile [10]. In a previous report, it was demonstrated that PLGA nanoparticles provided controlled and effective delivery of BNIPDaoct for treatment of visceral leishmaniasis caused by *Leishmania infantum*

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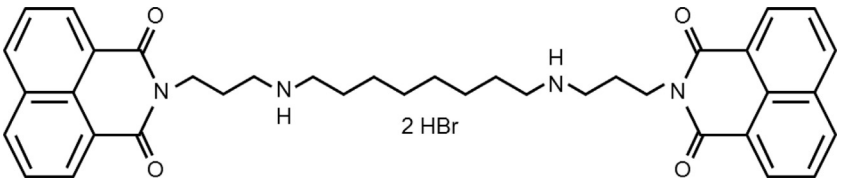

Fig. 1. Chemical structure of bisnaphthalimidopropyldiaaminooctane (BNIPDaoct).

protozoa [11]. In this respect it has emerged a need for rapid, sensitive and reliable analytical method that can be used to accurately quantify BNIPDaoct in biological samples.

Concerning the analytical determination of bisnaphthalimides, reports are scarce. A validated method for determination of bisnafide in human plasma by HPLC with UV detection has been described, requiring extensive sample treatment, involving removal of sample proteins, pH adjustment, extraction to ethyl ether followed by back-extraction to phosphoric acid aqueous solution [12]. In fact, fluorescent properties of bisnaphthalimides [13,14] have been applied for monitoring their binding to biomolecules [15], but their application in validated analytical methods have not been described yet.

In this context, HPLC coupled to fluorometric detection is a suitable tool to bioanalysis of nanoparticles loaded with bioactive compounds. In fact, C18 monolithic columns, consisting of micro- and mesopores [16,17], have been shown as suitable alternatives for bioanalysis [18,19], fostering minimal sample treatment. Hence, the objective of the present work was the development and validation of HPLC method based on monolithic column for determination of BNIPDaoct in biological samples, targeting the evaluation of its biodistribution as free compound or loaded in nanoparticles.

2. Experimental

2.1. Chemicals

Sodium acetate and octanesulfonic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile (LiChrosolv HPLC grade), dimethyl sulfoxide (DMSO) and acetic acid were obtained from Merck (Darmstadt, Germany). Water from Arium water purification system (resistivity > 18 MΩ cm, Sartorius, Göttingen, Germany) was used for the preparation of solutions.

PLGA (lactide:glycolide [65:35], molecular weight: 40,000–75,000 Da) and poly(vinyl alcohol) (PVA; 87–89% hydrolyzed, molecular weight: 13,000–23,000 Da) were acquired from Sigma–Aldrich. BNIPDaoct (containing 2HBr, molecular weight of 780.5 Da), represented in Fig. 1, was synthesized as described previously [4].

2.2. Preparation and characterization of PLGA nanoparticles containing BNIPDaoct

The biodegradable and biocompatible PLGA was used for the production of the nanoparticles by a nanoprecipitation method described in detail elsewhere [11]. Briefly, the polymer was dissolved in acetone at ~10 mg mL^{-1} to form the diffusing phase. BNIPDaoct in DMSO was then added to reach 10% drug loading (w/w). This phase (c.a. 1 mL) was added to the PVA 1% (w/v) dispersing phase (10–15 mL) and the organic solvent was evaporated overnight, at room temperature. The formed nanoparticles were then recovered and washed by centrifugation, resuspended in phosphate buffered saline (PBS) at pH 7.4. Unloaded PLGA nanoparticles were also prepared, using the same procedure without the addition of BNIPDaoct.

Particle size and distribution (polydispersity index, PI) were determined by dynamic light scattering (DLS), using a Zetasizer Nano ZS laser scattering device (Malvern Instruments Ltd., Malvern, UK) as described elsewhere [11].

2.3. Chromatographic analysis

2.3.1. Equipment and analytical conditions

Samples were injected (20 μL) into a reversed-phase monolithic column (Chromolith RP-18e, 100 mm × 4.6 mm i.d., Merck), connected to a Jasco (Easton, USA) HPLC system (pump PU-2089, autosampler AS-2057 and LC-Net II/ADC controller) coupled to a fluorimetric detector (Jasco FP-2020, λ_{exc} = 234 nm, λ_{em} = 394 nm). The chromatographic separation was achieved by isocratic mode using a mobile phase consisting of aqueous buffer (acetic acid/acetate 0.10 M, pH 4.5, 0.010 M octanesulfonic acid)-acetonitrile (60:40, v/v) at a flow rate of 1.5 mL min^{-1}. Mobile phase was filtered through a 0.22 μm Millipore GVWP filter. Prior to use, the mobile phase was degassed in an ultrasonic bath for 15 min. Determinations were performed at room temperature (20 ± 2 °C).

2.3.2. Preparation of stock and standard solutions

Stock solutions of BNIPDaoct were prepared daily in mobile phase at 20 μM, followed by intermediate dilution to 1 μM. Working standards were prepared from 1 μM intermediate stock solution, ranging from 2 to 100 nM. QC samples at three different levels (low, middle and high) were prepared (6, 20 and 100 nM) in mobile phase from the intermediate stock solution.

2.3.3. Preparation of biological matrices samples

Firstly, methanol was added to each organ at 1:2 (w/v, g/mL) for kidney, liver, and lung or at 1:5 (w/v, g/mL) for heart and spleen, following homogenization using a high-intensity IKA ultra-turrax. Next, the homogenates were dried under N_2 and then they were reconstituted using mobile phase (1:5, w/v, g/mL). This mixture was vigorously vortexed for 30 s, followed by sonication during 1 min, and then vortexed again for 30 s. The extract obtained was filtered (PVDF, 0.22 μM) and analyzed for BNIPDaoct content by HPLC. QC samples were also prepared using the extract, as described in Section 2.3.2.

Six-week-old male BALB/c mice were used (Charles River, Barcelona, Spain). Animals were housed five per cage for acclimatization one week before the experiments at the animal resource facilities of the IBMC (Porto, Portugal). All experiments were approved by and conducted in accordance with the IBMC/INEB Animal Ethics Committee and the Portuguese Veterinary Director General guidelines. Formulations containing BNIPDaoct in solution or nanoencapsulated (165 μM) were administered intravenous via the lateral tail vein (1.0 mg kg^{-1}) to each group of four healthy male BALB/c mice. The animals were sacrificed 1 h after BNIPDaoct injection with lethal dose of anaesthesia. The organs were collected and preserved in ice during all the procedure. The concentration of BNIPDaoct in the biological matrices (spleen, heart, liver, lungs, and kidneys) was determined by processing the samples as described above, with further assessment of BNIPDaoct content by the developed and validated HPLC method.