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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 bisnaphthalimidopropyldiaminoctane (BNIPDaoct), a potent anti-*Leishmania* compound, incorporated into poly(D,L-lactide-co-glycolic acid) (PLGA) nanoparticles was developed and validated towards bioanalysis application. Biological tissue extracts were injected into a reversed-phase monolithic column coupled to a fluorimetric detector (λexc = 234 nm, λemis = 394 nm), using isocratic elution with aqueous buffer (acetic acid/acetate 0.10 mol L^{-1}, pH 4.5, 0.010 mol L^{-1} 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 µmol L^{-1}. 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 nmol L^{-1}, 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.

Keywords: Poly(D,L-lactide-co-glycolic acid); Bisnaphthalimidopropyldiaminooctane; Anti-leishmanial compound; Monolithic column; Fluorometry

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* 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.

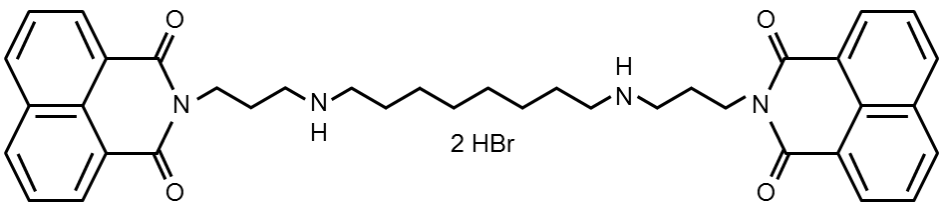


Figure 1. Chemical structure of BNIPDaoct

Concerning the analytical determination of bisnaphthalimides, reports are scarce. A validated method for the 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, Goettingen, Germany) was used for the preparation of solutions. Aqueous buffer (acetic acid/acetate 0.10 mol L^{-1}, pH 4.5, 0.010 mol L^{-1} octanesulfonic acid) 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.

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, represented in Fig. S1, was synthesized as described previously [4].

2.2. Preparation and characterization of PLGA nanoparticles containing BNIPDaoct

The biodegradable and biocompatible PLGA was chosen 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 compound.

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]. Their mean size was 156 ± 3 nm, with a polydispersity index of 0.08 ± 0.03, and Zeta potential of -5.1 ± 0.7 mV.

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 mol L^{-1}, pH 4.5, 0.010 mol L^{-1} octanesulfonic acid)-acetonitrile (60:40, v/v) at a flow rate of 1.5 mL min^{-1}.

2.3.2. Preparation of stock and standard solutions

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

2.4. Method validation

The chromatographic method was validated for specificity, linearity, accuracy, precision, range and robustness in accordance with EMA and ICH guidelines [20, 21].

2.4.1. Selectivity

Selectivity of the method was determined by analyzing six blanks of each matrix, including mobile phase, heart, kidney, liver, lung or spleen obtained from healthy mice.

2.4.2. Linearity and calibration range

147 To evaluate linearity, calibration curves were prepared and analyzed in triplicate,
148 comprising three independent experiments. Data were fitted to least squares linear
149 regression concerning peak area vs. concentration for ten standards prepared in mobile
150 phase (2, 4, 6, 8, 10, 20, 40, 60, 80 and 100 nmol L^{-1}) or six standards prepared in each
151 biological matrix (2, 6, 10, 20, 60 and 100 nmol L^{-1}), and by analysis of the respective
152 response factors (i.e., peak area divided by concentration of each standard sample).
153 Back calculated concentrations were also obtained [20].

154 *2.4.3. Accuracy and precision*

155 The accuracy and precision were determined by analyses of QC samples at three
156 concentration levels (6, 20 and 100 nmol L^{-1}). The accuracy was expressed by percent
157 of the nominal concentration value ((mean measured concentration)/(nominal
158 concentration) × 100%) and the precision by the coefficient of variation (standard
159 deviation/mean) × 100%). Intra-day (within-run) values were obtained by replicate
160 analyses (n = 6) followed by interpolation in calibration curves prepared on the same
161 day. Inter-day (between-run) values were obtained from 3 independent experiments (n =
162 3).

163 *2.4.4. Limit of detection and quantification*

164 The limits of detection (LOD) and quantification (LOQ) for each matrix were calculated
165 as $f \times \sigma / S$, where f is 3 (LOD) or 10 (LOQ), σ is the statistics $s_{y/x}$, and S is the slope of
166 the calibration curve [22].

167 *2.4.5. Robustness*

168 The short-term stability was assessed by maintaining the QC samples at RT for 24 h.
169 Freeze-thaw stability of the samples was obtained over three freeze-thaw cycles, by
170 thawing at RT for 2 h and refreezing for 24 h. Autosampler stability of BNIPDaoct was
171 tested by analysis of QC samples, which were stored in the autosampler tray of the

HPLC instrument for 24 h. For each concentration and each storage condition, six replicates were analyzed in one analytical batch. The concentration of BNIPDaoct after each storage period was compared to the initial concentration determined for the fresh samples, processed immediately after preparation.

2.5. Recovery of BNIPDaoct from biological tissue

Extractions for the recoveries of BNIPDaoct from biological matrices at three QC levels were performed by spiking the organ extract (section 2.4.2) in mobile phase or by spiking the organ tissue before extraction. Recovery and precision were determined as described in Section 2.4.3.

2.6. Application to biodistribution studies

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 in section 2.3.3, with further assessment of BNIPDaoct content by the developed and validated HPLC method.

3. Results and discussion

3.1. Development of chromatographic method

Considering the method application to studies of biodistribution and pharmacokinetics, the main goal to be achieved in the development of this chromatographic method was low detection and quantification limits, using low sample volumes. Furthermore, as a high sample throughput was aimed, a monolithic column coupled to fluorescence detection was chosen to meet these needs.

Moreover, the development of a simplified HPLC method with isocratic elution was aimed. Acetonitrile was chosen as organic component and the aqueous component was composed by 0.10 mol L^{-1} acetic acid/acetate buffer, pH 4.5 and 0.010 mol L^{-1} octanesulfonic acid in order to guarantee pH control (buffer) and to avoid peak tailing (sulfonate as ionic pairing agent). The effect of aqueous to organic ratio was investigated using 0.05 µM BNIPDaoct standard. As expected, when the percentage of the organic phase increases, the retention time of BNIPDaoct decreases. For example, when increasing the organic phase from 35 to 50% (v/v), the retention time decreased to 20.3%. In fact, for intermediate percentages of 40 and 45% (v/v), the retention time was 39.2 and 25.0% of that observed for 35% (v/v) of acetonitrile. Hence, in order to attain a fast separation while maintaining good separation of BNIPDaoct from the void volume signal (non-retained matrix components), the proportion of aqueous to organic components in the mobile phase was fixed at 60:40 (v/v).

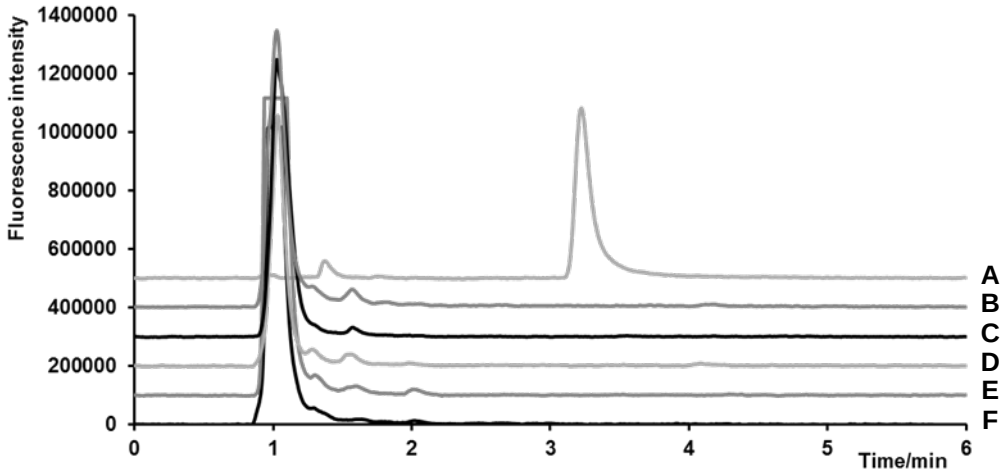
Considering that monolithic columns can provide analyte separations using high flow rates with an increase of pressure that can be withstand by any HPLC system, the flow rate applied was studied, in order to maximize sample throughput and minimize mobile phase consumption. The retention time decreased to 25.7, 33.7 and 50.2% when applying 2.0, 1.5, and 1.0 mL min^{-1}, compared to the value obtained for 0.5 mL min^{-1}.

222 Hence, a flow rate of 1.5 mL min^{-1} was selected, considering that it afforded low
223 acetonitrile consumption and a pressure of 2.5 MPa within the HPLC system. The run
224 time was 6 min, with a retention time of 3.3 min for BNIPDaoct compound.

225 *3.2. Method validation and application to biological samples*

226 The validation of the analytical HPLC method was carried out by evaluating its
227 selectivity, linearity, intra-day and inter-day precision and accuracy, stability, and
228 extraction recovery.

229 For selectivity, analyses of blank samples of all the matrices were performed as shown
230 in Fig. 2, where a chromatogram for 0.10 µmol L^{-1} BNIPDaoct standard is also
231 presented. No interference for BNIPDaoct detection from endogenous compounds
232 present in tissue extracts was observed as no peaks at the BNIPDaoct retention time
233 were detected (Fig. 2). Any potential interfering compound was washed from the
234 column together with the solvent front, particularly for liver and kidney sample



235

236 Figure 2. Chromatograms of BNIPDaoct (0.10 µmol L^{-1}, A) and blank heart (B), spleen
237 (C), lung (D), kidney (E), and liver (F).
238

239 Regression analysis data from calibration curves in mobile phase and biological tissue
240 extracts are given in Table 1. Two calibration ranges were established for BNIPDaoct: a
241 low range for concentrations between 0.002 and 0.020 µmol L^{-1} and a high range

between 0.010 and 0.100 µmol L^{-1}. Sensitivity, assessed as the slope of the calibration curve, was 5 to 10% lower for the low range. Calibration curves for BNIPDaoct in mobile phase were linear and reproducible, with correlation coefficient > 0.9996. The back calculated concentrations presented deviations < 5.9% from the nominal value, meeting the requirements of EMA guideline [20]. The calculated LOD was 0.5 nmol L^{-1} and the LOQ was 1.6 nmol L^{-1}.

Calibration curves for BNIPDaoct in the biological matrices tested were also linear for the two ranges correlation coefficients ≥ 0.9997 (Table 1). The LOD and LOQ for biological matrices were ≤ 0.8 and ≤ 1.8 nmol L^{-1}, respectively (Table 1), corresponding to values ≤ 4 and ≤ 9 nmol g^{-1} in mice organs.

Table 1. Calibration curve parameters[a], limit of detection (LOD) and quantification (LOQ) for BNIPDaoct in mobile phase and tissue extracts.

Matrix	Range (µmol L^{-1})	Slope (L µmol^{-1})	Intercept	LOD (µmol L^{-1})	LOQ (µmol L^{-1})
Mobile phase	0.002 – 0.020	4.837×10^7	7299	0.0005	0.0016
	0.010 – 0.100	5.269×10^7	-33879	---	---
Spleen	0.002 – 0.020	4.932×10^7	3291	0.0003	0.0011
	0.010 – 0.100	5.118×10^7	-15036	---	---
Heart	0.002 – 0.020	4.931×10^7	4488	0.0002	0.0010
	0.010 – 0.100	5.111×10^7	-12331	---	---
Liver	0.002 – 0.020	4.918×10^7	-16174	0.0008	0.0018
	0.010 – 0.100	5.307×10^7	-60002	---	---
Lungs	0.002 – 0.020	4.838×10^7	-494	0.0004	0.0015
	0.010 – 0.100	5.144×10^7	-18625	---	---
Kidneys	0.002 – 0.020	4.778×10^7	-974	0.0005	0.0016
	0.010 – 0.100	5.028×10^7	-17567	---	---

[a] R > 0.9996 for all calibrations.

Accuracy and precision (inter- and intra-day) were also estimated. The intra-day precision and accuracy were calculated by analyzing the QC of all matrices at concentrations of 0.006, 0.020 and 0.100 µmol L^{-1} and the 0.020 µmol L^{-1} standard was

interpolated in both calibration curves (Table 2). The intra-day precision was ≤ 1.9% and accuracy ranged between 97.3 and 106.8%. The inter-day precision was ≤ 7.2% and accuracy ranged between 96.6 and 105.6% (Table 2). Both intra- and inter-day precision and accuracy found have acceptable values, because precision for each concentration level, represented as CV, did not exceed 15% and the accuracy range was between 85 and 115% [20].

Table 2. Accuracy and precision for the analysis of BNIPDaoct in mobile phase and tissue extracts.

		Intra-day			Inter-day		
Matrix	Nominal concentration [a] (µmol L^{-1})	Measured concentration			Measured concentration		
		Mean (µmol L^{-1})	Accuracy (%)	CV (%)	Mean (µmol L^{-1})	Accuracy (%)	CV (%)
Mobile phase	0.006	0.0058	97.3	1.4	0.0058	96.6	5.4
	0.020 (LL)	0.0202	101.1	0.7	0.0204	101.8	1.4
	0.020 (HL)	0.0200	100.2	1.5	0.0199	99.9	0.6
	0.100	0.1001	100.1	1.6	0.1001	100.1	1.0
Spleen	0.006	0.0061	101.6	1.9	0.0061	102.0	1.7
	0.020 (LL)	0.0199	99.7	0.5	0.0200	100.1	0.4
	0.020 (HL)	0.0198	99.2	0.6	0.0199	99.5	0.5
	0.100	0.1002	100.2	0.2	0.1001	100.1	0.3
Heart	0.006	0.0060	100.7	0.7	0.0061	101.1	0.6
	0.020 (LL)	0.0199	99.3	0.1	0.0200	100.0	0.6
	0.020 (HL)	0.0198	99.0	0.2	0.0199	99.4	0.5
	0.100	0.1004	100.4	0.1	0.1004	100.4	0.1

In order to evaluate the robustness of the developed method, stability and recovery assays were performed. The stability of BNIPDaoct at laboratory temperature was assessed analyzing fresh samples and analyzing the same samples after 24 h at RT (20 ± 2 °C). BNIPDaoct showed stability in all matrices after this period as can be seen from data in Table 3, where recoveries ranged from 92.6 to 105.9%. The autosampler stability was assessed analyzing fresh samples and analyzing the same samples after 24 h after the first injection. BNIPDaoct was stable in the autosampler for at least 24 h (Table 3), providing recovery values in all matrices between 94.1 and 103.2%. The freeze–thaw stability of BNIPDaoct over three freeze–thaw cycles was also assessed, providing values of 89.1-102.5% for analyte recovery (Table 3), showing the stability during sample storage and handling.

Table 3. Stability of BNIPDaoct at different experimental conditions for all matrices tested.

Experimental condition		24 h at room temperature			24 h in the autosampler			3 freeze-thaw cycles		
Matrix	Nominal concentration[a] (µmol L^{-1})	Measured concentration			Measured concentration			Measured concentration		
		Mean (µmol L^{-1})	Accuracy (%)	CV (%)	Mean (µmol L^{-1})	Accuracy (%)	CV (%)	Mean (µmol L^{-1})	Accuracy (%)	CV (%)
Mobile phase	0.006	0.0057	94.3	0.8	0.0053	88.5	2.1	0.0057	95.6	2.5
	0.020 (LL)	0.0201	100.6	0.6	0.0191	95.5	1.4	0.0201	100.5	1.0
	0.020 (HL)	0.0197	98.4	0.5	0.0193	96.7	0.5	0.0195	97.4	1.5
	0.100	0.0993	99.3	1.3	0.0947	94.7	3.7	0.0973	97.3	0.6
Spleen	0.006	0.0061	100.9	1.5	0.0058	96.6	2.4	0.0058	97.0	1.0
	0.020 (LL)	0.0197	98.7	0.5	0.0195	97.5	0.4	0.0198	99.0	0.4
	0.020 (HL)	0.0197	98.3	0.5	0.0194	97.0	0.4	0.0195	97.7	0.4
	0.100	0.0999	99.9	0.1	0.0976	97.6	0.7	0.0979	97.9	0.1
Heart	0.006	0.0059	98.7	0.6	0.0059	97.6	0.9	0.0058	96.4	1.4
	0.020 (LL)	0.0199	99.3	0.3	0.0197	98.4	0.3	0.0198	99.2	0.2
	0.020 (HL)	0.0198	99.0	0.4	0.0197	98.2	0.3	0.0197	98.6	0.1
	0.100	0.0983	98.3	0.1	0.0983	98.3	0.1	0.0984	98.4	0.1

281 Finally, extraction efficiency was assessed, by spiking organs harvested from healthy
282 animals and processing as described in section 2.3.3 (Table 4). Recoveries were similar
283 to all tissues and for both BNIPDaoct in solution (mean recovery 93.2 ± 2.1%) and
284 nanoencapsulated BNIPDaoct (mean recovery 92.3 ± 2.4%). Therefore, recovery values
285 are acceptable for all organs and suitable for reliable bioanalysis.

286 **Table 4.** Extraction efficiency of BNIPDaoct from mouse organs at different
287 concentration levels

		BNIPDaoct solution			BNIPDaoct nanoparticles		
Matrix	Target concentration on extract (μmol L^{-1})	BNIPDaoct:organ mass ratio	Recovery (%)	CV (%)	BNIPDaoct:organ mass ratio	Recovery (%)	CV (%)
Spleen	0.006	2.3×10^{-8}	94.7	1.6	2.3×10^{-8}	87.5	1.1
	0.020 (LL)	7.8×10^{-8}	93.9	0.6	7.8×10^{-8}	95.1	0.2
	0.020 (HL)	7.8×10^{-8}	94.0	0.8	7.8×10^{-8}	95.4	0.5
	0.100	3.8×10^{-7}	90.0	0.1	3.9×10^{-7}	87.6	0.1
Heart	0.006	2.3×10^{-8}	93.3	0.8	2.4×10^{-8}	96.1	1.4
	0.020 (LL)	3.1×10^{-8}	90.8	0.8	7.8×10^{-8}	91.2	1.0
	0.020 (HL)	3.1×10^{-8}	90.2	0.3	7.8×10^{-8}	91.3	0.8
	0.100	1.6×10^{-7}	90.6	0.2	3.9×10^{-7}	89.0	0.1
Liver	0.006	1.2×10^{-8}	93.7	1.4	9.4×10^{-9}	92.4	1.0
	0.020 (LL)	3.1×10^{-8}	95.3	1.4	3.1×10^{-8}	93.3	0.7
	0.020 (HL)	3.1×10^{-8}	94.2	0.7	3.1×10^{-8}	94.4	1.1
	0.100	1.7×10^{-7}	94.0	0.3	1.6×10^{-7}	92.2	0.3

288

289 **Table 4.** Extraction efficiency of BNIPDaoct from mouse organs at different
290 concentration levels (continuation).

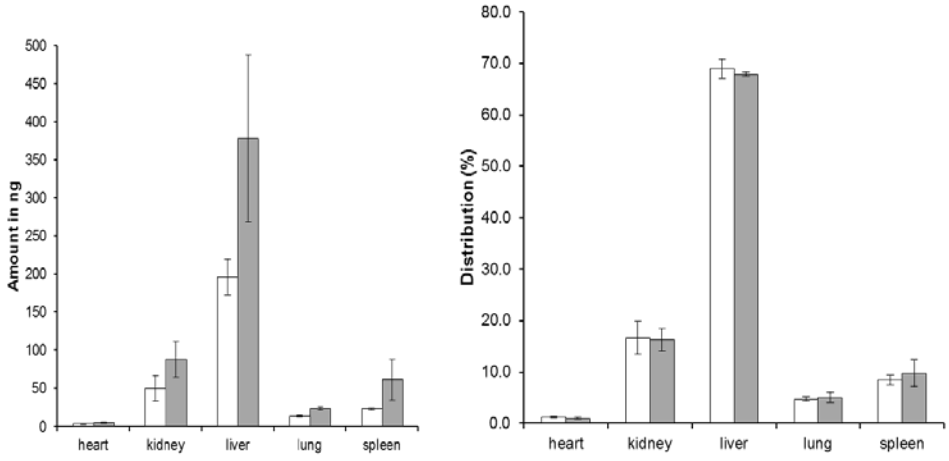
		BNIPDaoct solution			BNIPDaoct nanoparticles		
Matrix	Target concentration on extract (μmol L^{-1})	BNIPDaoct:organ mass ratio	Recovery (%)	CV (%)	BNIPDaoct:organ mass ratio	Recovery (%)	CV (%)
Lungs	0.006	9.2×10^{-9}	93.4	0.9	9.4×10^{-9}	91.9	1.5
	0.020 (LL)	3.1×10^{-8}	95.0	1.1	3.1×10^{-8}	92.7	0.7
	0.020 (HL)	3.1×10^{-8}	95.5	0.7	3.1×10^{-8}	93.1	0.8
	0.100	1.6×10^{-7}	93.2	0.2	1.6×10^{-7}	92.2	0.2
Kidneys	0.006	9.4×10^{-9}	91.1	0.8	9.4×10^{-9}	92.4	1.0
	0.020 (LL)	3.1×10^{-8}	92.0	0.1	3.1×10^{-8}	93.3	0.7
	0.020 (HL)	3.1×10^{-8}	91.4	0.3	3.1×10^{-8}	94.4	1.1
	0.100	1.6×10^{-7}	98.3	0.1	1.6×10^{-7}	92.2	0.3

291

292 *3.3. Application to biodistribution assessment*

293 The validated HPLC method was applied to assess the biodistribution of free and
294 nanoencapsulated BNIPDaoct. The amount found in each organ and the relative
295 distribution is given in Fig. 3. A larger amount of BNIPDaoct (3.1% of administered
296 mass as free drug) was found compared to BNIPDaoct administered in
297 nanoencapsulated form (1.5% of administered mass). The distribution per harvested
298 organs was similar for both formulations, with a large amount found in liver (68%), and
299 followed by kidney (16%), spleen (10%), lung (5%) and heart (1%). Considering the
300 organs were harvested 1 hour after drug administration, a similar distribution profile
301 was observed, with a larger amount of free drug recovered. Nevertheless, we would
302 expect an increased amount of encapsulated BNIPDaoct if quantification was performed
303 following a longer time (e.g. 24 h) after drug administration because the bio-

accumulation of encapsulated BNIPDaoct requires more time due to their interactions with complement system and inespecific cellular interactions.



A B

Figure 3. Amount (A) and relative distribution (B) of BNIPDaoct recovered from mice organs (n = 4) after administration of nanoencapsulated (white bars) or free drug (black bars).

4. Conclusions

A simple, sensitive, accurate and precise HPLC method was developed and fully validated for determination of BNIPDaoct in biological samples. This method was convenient for the quantification of BNIPDaoct in mice organ samples and it was successfully applied to the evaluation of biodistribution of BNIPDaoct in mice. Further application will target pharmacokinetic studies and application of other formulations for BNIPDaoct delivery. Furthermore this validated method can potentially be applied in the biodistribution of other mono and bisnaphthalimido molecules.

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Figure Captions

Figure 1. Chemical structure of bisnaphthalimidopropyldiaaminooctane (BNIPDaoct).

Figure 2. Chromatograms of BNIPDaoct (0.10 μmol L^{-1}, A) and blank heart (B), spleen (C), lung (D), kidney (E), and liver (F).

Figure 3. Amount (A) and relative distribution (B) of BNIPDaoct recovered from mice organs (n = 4) after administration of nanoencapsulated (white bars) or free drug (black bars).