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ORIGINAL ARTICLE

Development of an HPLC Method for Identification and Quantification of Anti-leishmaniasis Drug Candidate NFOH After Oral Administration of NLC-NFOH in Rats

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Abstract
Hydroxymethylnitrofurazone (NFOH) is a prodrug synthesized from the original nitrofurazone compound (NF). NFOH has shown activity in vivo against *Trypanosoma cruzi* and in vitro activity against *Leishmania amazonensis*. However, it has shown less toxicity than NF. This study aims to develop and evaluate the selectivity, carryover, matrix effect, linearity, precision and accuracy, of a bioanalytical method using HPLC to quantify NFOH and NF. The defined method conditions were mobile phase acetonitrile: water (20:80 *v/v*), Zorbax SB-C18, 5 µm (4.6 × 250 mm) flow rate of 1.2 mL min^{-1}, at UV detection of 370 nm. The linearity obtained for NFOH and NF was in the range of 0.025–3.0 µg mL^{-1} with a correlation coefficient > 0.98. The precision (relative standard deviation) was 2.44–13.77% and 2.61–18.42% for NFOH and NF, respectively; the accuracy was 2.66–14.28% and 2.09–19.06% for NFOH and NF, respectively. In conclusion this method was successfully developed to determine and quantify NFOH and NF in serum after oral administration of nanostructured lipid carrier with NFOH. It opens opportunities to be applied in in vivo studies in novel NFOH nanoparticles formulations.

Keywords Bioanalytical method · HPLC · Leishmaniasis · Lymphatic system · Nanostructured lipid carrier

1 Introduction

Hydroxymethylnitrofurazone (NFOH) (Fig. 1A) was first obtained by Chung et al. [1] using a molecular modification of the original nitrofurazone compound (NF) (Fig. 1B). NF is an antimicrobial agent primarily active against Gram-positive microorganisms and used only in topical infections due to its adverse effects. Due to the low aqueous solubility and high toxicity of NF, a prodrug, NFOH, was developed to overcome these challenges. NFOH showed activity against *Trypanosoma cruzi* and less toxicity than NF [1]. Moreover, it showed in vitro activity against *Leishmania amazonensis* [2]. The pharmacokinetic studies showed that NFOH converted into NF after hydrolysis in in vitro and in vivo studies [3].

Leishmaniasis is a neglected disease and presents two primary clinical forms: cutaneous leishmaniasis and visceral leishmaniasis [4]. This is a disease transmitted by the bite of female phlebotomine sandflies. After the bite, the promastigote form of the parasite infects dendritic cells and macrophages, and the parasite passes to the amastigote form. The amastigotes forms lead to the rupture of those cells, disseminating the infection [5].

Several studies have demonstrated different ways to treat Leishmaniasis. And the nanostructured systems have presented promising results. Among them there are nanometallic [6, 7], nanopolymeric [2] and nanolipid systems [8]. In the present study we chose nanostructured lipid carriers (NLCs) to load NFOH, since they are known to demonstrate excellent performance as modified drug delivery systems. The advantages of the development of NLCs are low production cost, no need to use organic solvents,

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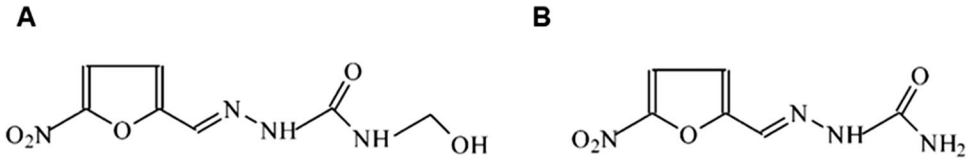

Fig. 1 Chemical structure of NFOH (**A**) and NF (**B**)

and high stability. In addition, they have shown reduced toxicity, encapsulation efficacy of lipophilic molecules, and being site-specific [9, 10]. NLCs are composed of colloidal particles that present a matrix composed of a binary mixture of solid lipid and liquid lipid, resulting in a less ordered structure, which prevents the risk of an expulsion of the drug during shelf-life [10]. Studies have demonstrated that NLCs can improve the efficacy of the leishmaniasis treatment [11–13].

Some studies have been used to quantify NF by HPLC [14–17]. Tubino et al. used a µBondapac C18 (300 mm × 3.9 mm, 10 µm) column, flow rate of 2 mL min^{-1}, at 365 nm to quantify NF [14]. The mobile phase was composed of water, acetonitrile and triethylamine buffer in the 790:200:10 *v/v/v*. The retention time was 8 min [14]. Another work has described a forced-degradation method for NF by HPLC using a Zorbax Eclipse XDB–C18 (4.6 × 150 mm, 5 µm) column and mobile phase of 0.025 M disodium hydrogen phosphate, methanol, and triethylamine, 70:30:0.1 (*v/v/v*)—pH 4.0. The flow rate was 1.0 mL min^{-1} at 374 and 220 nm, and retention time was 4.5 min [18]. Monteiro et al. has described a NFOH analysis using Hypersyl C18 (5 µm, 4.6 × 150 mm) and mobile phase of water and acetonitrile, 80:20 (*v/v*), flow rate at 1.2 mL min^{-1}, at 265 nm. The retention time was 4.9 min [19].

The quantification of NFOH have been performed by different authors [19–21]. Nogueira Filho et al. used a LCMS/MS to quantify NFOH in male rabbits' plasma after oral administration of NFOH solution with DMSO. The method as validated using FDA Guidance for Industry Bioanalytical Method Validation. The range for linearity was 0.25–10 µg/mL. Diclofenac in methanol was used as an internal standard and methanol:water (50:50 v/v) as mobile phase. Sample preparation was done by adding ethyl acetate, vortexing and centrifuging, then the supernatant was evaporated and resuspended with the mobile phase [20].

Another study involving the quantification of NFOH in biological material by HPLC, does not describe in detail the sample preparation. In addition, there is no description of validation or analytical evaluation of the figures of merit [3].

However, one requires a bioanalytical method for identification and quantification of NFOH loading in NLCs system in serum. So, this study aims to develop and evaluate analytical figures of merit of a sensitive and simpler bioanalytical method using HPLC–UV.

2 Material and Methods

2.1 Material

NFOH and NF were obtained by synthesis [1]. Acetonitrile HPLC grade was purchased from JT Baker. Purified water was prepared using a Milli-Q water purification system (Millipore, USA). All other reagents were analytical grade. NLC-NFOH was prepared as described by De Souza et al. [21].

2.2 Apparatus and Chromatographic Conditions

The chromatographic system was a Shimadzu HPLC–UV system which consisted of a CBM-20A controller, LC-20AT-pump, SPD-20A detector, and SIL-20AC sampler, and LC Solution software (version 1.25SP4) was applied for data collecting and processing. Chromatographic separation was performed on a Zorbax SB-C18, 5 µm, (4.6 × 250 mm) HPLC column. The mobile phase consisted of acetonitrile:water (20:80, *v/v*) at a flow rate of 1.2 mL min^{-1} and UV detector at 370 nm. The volume of injection was 20 µL.

2.3 Preparation of Standard Stock Solution, Working Standard Solution

Standard stock solutions of NFOH and NF (100 µg mL^{-1}) were prepared in acetonitrile. The solutions were sonicated for 10 min and kept under refrigeration at 5 °C until analysis. Working standard solution was freshly prepared daily by appropriately diluting the stock solutions with water. The final concentration of standard NFOH and NF solution was 5.0 µg mL^{-1}.

2.4 Sample Extraction

Acetonitrile (200 µL) was added to rat serum samples (100 µL) to precipitate proteins. This solution was then vortexed for one min, centrifuged at 16,595 g for 20 min, and the resultant supernatant was analyzed in HPLC.

2.5 Bioanalytical Method Development

The Monteiro et al. [19] method was modified and adjusted to provide separation among NFOH, NF and interferents

from serum. Subsequently, 200–600 nm screening using a UV–vis spectrophotometer (Evolution series 201, Thermo Scientific Inc. USA) was performed to define the best wavelength.

2.6 HPLC Method Validation

Method validation was based on the Bioanalytical Method Validation Guidance for Industry from the Food and Drug Administration [22].

2.6.1 Selectivity

Selectivity was evaluated by the analysis of the presence of interferent in the blank matrix at the same retention time as NFOH and NF.

2.6.2 Carryover

During the validation, the carryover evaluated the alteration of a measured concentration due to residual analyte from a preceding sample. Two injections of the blank sample were run before the upper limit of quantification (ULOQ) and two injections after ULOQ.

2.6.3 Matrix Effect Factor

The low-quality control (LQC) and high-quality control sample (HQC) concentration levels were evaluated in water in triplicate, compared to the serum matrix. Equation 1 shows the matrix effect factor (MEF); the RSD of the MEF was used to calculate the matrix effect.

$$\mathrm{MEF} = \frac{\text{Analyte response at serum}}{\text{Analyte rsponse at water}} \tag{1}$$

2.6.4 Linearity and LLOQ

The linearity of the method was evaluated at seven different concentrations in triplicate, ranging from 0.025 to 3.0 µg mL^{-1} for NFOH and NF to obtain the linear curve in serum. The solutions were freshly prepared by appropriately diluting the stock solutions with water. The regression parameters were analyzed statically in accordance to the Bioanalytical Method Validation Guidance for Industry [22]. The lower limit of quantification (LLOQ) was considered the lowest concentration of the calibration standard.

2.6.5 Accuracy and Precision

The method was evaluated for accuracy and precision by analyzing LLOQ, LQC, medium quality control (MQC), and HQC concentration levels within the calibration curve (0.025 µg mL^{-1}; 0.25 µg mL^{-1}; 1.0 µg mL^{-1}; 2.5 µg mL^{-1}) in serum. Five replicates were analyzed on the same day for intra-day analysis. Precision was expressed as percent relative standard deviation (RSD) (Eq. 2) and the accuracy as percent recovery (Eq. 3).

$$RSD = \frac{Standard\ deviation}{Experimental\ average\ concentration} \times 100 \tag{2}$$

$$\%\ Recovery = \frac{(Experimental\ average\ concentration - theoretical\ value)}{Teoretical\ value} \times 100 \tag{3}$$

2.7 Application

Male Wistar rats (50–60 days) were kept at 23 ± 2 °C in a 12 h light–dark cycle, with free access to food and water. All rats received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care, and the Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, published by the National Institute of Health (NIH Publication No. 86–23, revised 1996). The Ethics Committee approved the animal experiment protocol (protocol n°1155/2019). All rats were fasting the night before the administration of the formulation.

After 1 h oral administration of NLC-NFOH (2.8 mg kg^{-1}) by gavage, the animals were anesthetized with isoflurane (5% and 95% respectively) in a closed glass chamber, and subsequently maintained with isoflurane and compressed air (2% and 98% respectively) using an anesthesia mask. They were submitted to euthanasia through total blood exsanguination. Blood samples were centrifuged at 1258*g* for 15 min (Eppendorf® Centrifuge 5810R), and the serum was stored at − 20 °C until further analysis by HPLC.

3 Result and Discussion

3.1 Development of Method

For the quantification of NFOH and NF in serum, it is important to develop a sensitive and selective bionalytical method. Due to the complexity of biological tissue, the pretreatment of matrix is a relevant step for the development. The purpose of pretreatment of biological matrices is to remove proteins, nucleic acids, and peptides [23]. Protein precipitation is one technique used to remove the complex biogenic compounds that can interact with the drugs [24].

For this work, acetonitrile was used to precipitate the proteins. Different proportions of serum and acetonitrile (1:1; 1:2; 1:3 and 1:4) were used to achieve satisfactory chromatographic behavior. The precipitation of proteins prevents clogging and the deterioration of the HPLC column. However if there is a high acetonitrile volume, the resolution of the peaks might be compromised, and the drug may be diluted, impairing its detection. After some tests, the proportion chosen was 1:2 (serum:acetonitrile).

The study of wavelength can improve the selectivity of the method, knowing that the short-wavelength can present poorly selective UV detection in biological tissue [25]. Thus, the method development by Monteiro et al. was adapted [19]. A UV–Vis spectrophotometry test was performed to determine a better wavelength (370 nm) due to the interferents at the same retention time of NFOH and NF at 265 nm (Fig. 2).

Another parameter evaluated was the injection volume (20 and 50 µL). Due to the use of acetonitrile in sample solution preparation, the high injection volume altered the resolution of the peaks. This phenomenon occurs because the higher strength of the organic solvent compared to the mobile phase leads to a portion of the sample moving more quickly through the column, forming wide peaks [26]. Thus, the volume of 20 µL of injection was selected.

The chromatography separation of NFOH and NF used acetonitrile and water (20:80, *v/v*) as mobile phase and flow of 1.2 mL min^{-1}. The retention times of the substances were 4.66 and 5.25 min for NFOH and NF, respectively.

3.2 HPLC Method Validation

After defined the chromatographic conditions, the following analytical figures of merit were evaluated: selectivity, carryover, matrix effect, linearity, LLOQ, accuracy, and precision.

Selectivity evaluates the ability of the method to differentiate the analyte in the presence of the biological matrix blank. Figure 3 shows there are no interferents in the retention times of NFOH and NF.

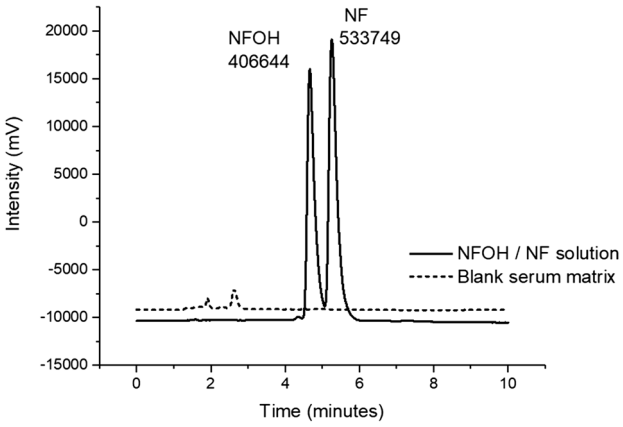

Fig. 3 Chromatogram of NFOH and NF standards solution (5.0 µg mL^{-1}) and blank serum matrix. Chromatographic conditions: Zorbax SB-C18, 5 µm, (4.6×250 mm) HPLC column; mobile phase—acetonitrile:water (20:80), flow at 1.2 mL min^{-1}; wavelength = 370 nm; volume of injection = 20 µL

Carryover is the effect generated by the appearance or increase of the analyte caused by contamination from previous runs. For NFOH, there was no carryover. Thus, it was possible to use the LLOQ for NFOH quantification analyzes in serum. The same did not occur with NF, which showed a carryover effect, influencing its quantification in the samples. This may have occurred because NF is more apolar (Log P_{NF} 0.23), than NFOH (Log P_{NFOH}—0.19), requiring washing during the run to reduce the carryover [1]. The method is intended to quantify the NFOH, since the oral administration in this study is made of an NLC with NFOH.

The MEF is an alteration in response due to the presence of interferent substances in the sample. The percent RSD of the MEF for all samples must not exceed 15%

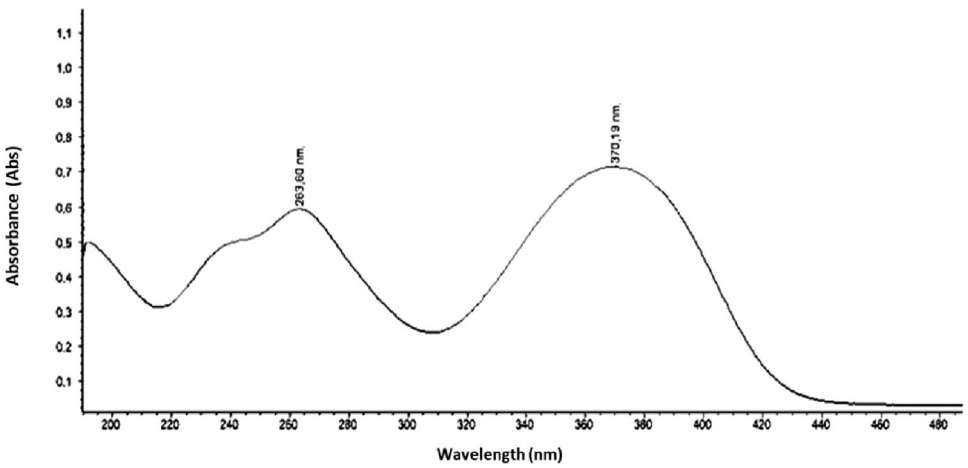

Fig. 2 Maximum NFOH absorbance of 10 µg mL^{-1} in the 200–500 nm range

(FDA, 2018). The RSDs found for LQC (0.25 µg mL^{-1}) were 13.07% and 6.26% for NFOH and NF, respectively. Moreover, RSDs found for HQC (2.50 µg mL^{-1}) were 7.39% and 13.73% for NFOH and NF, respectively.

The analytical curve was built using seven data points in a triplicate injection of matrix spiked with NFOH and NF. The curve range was 0.025–3.0 µg mL^{-1} for NFOH and NF with good linearity ($r > 0.98$) using a weighting of $1/x^2$. The results are presented in Table 1. The analysis of variance ($p < 0.05$, $\alpha = 0.5$) for NFOH and NF are showed in the supplementary material (Table S1 and Table S2). The evaluation of the regression efficiency showed the F-value greater than the tabulated for NFOH and NF. The analysis of lack of fit (error associated with the model) shows the error is not associated with the model, with $p > 0.05$, and F-value lower than the $F_{tabulated}$, for both substances.

Precision is the proximity of results obtained by repeated measurements in different concentrations from the same matrix. Furthermore, the accuracy is evaluated by the proximity of the values between the test and the reference [22]. According to the FDA, the acceptance criteria are 15% for RSD or %Recovery for all concentrations, except for LLOQ (0.025 µg mL^{-1}) which is accepted at 20%. The method showed to be in accordance with the legislation (Table 2).

3.3 Application

NLC-NFOH was administered by gavage to rats to evaluate the concentration of NFOH in serum. The NLC is a nanolipid system with colloidal particles formed by liquid and solid lipid mixture [27]. The use of NLC is an alternative for the treatment of leishmaniasis due to the feasible preparation and non-toxic system. The application of the method for quantifying NFOH in rat serum after oral administration of NLC-NFOH showed a concentration of 0.029 ± 0.004 µg mL^{-1}. The chromatogram did not show interfering peaks, enabling quantification within the method range (Fig. 4).

Table 1 Linear regression for the calibration curve of NFOH and NF in rat serum

Parameter	Statistical data	
	NFOH	NF
Concentration range (µg mL^{-1})	0.025–3.00	0.025–3.00
Intercept	−4.2	93.7
Slope (S)	20,904	25,992
Coefficient of correlation (r)	0.98	0.98
Residual SD of regression line (σ)	4216.02	4570.16
LLOQ (µg mL^{-1})	0.025	0.025

LLOQ lower limit of quantification, *NFOH* hydroxymethylnitrofurazone, *NF* nitrofurazone

Table 2 Precision and accuracy of NFOH and NF at serum

	Concentration (µg mL^{-1})	Precision (RSD)	Accuracy (%Recovery)
NFOH	0.025	10.96	2.66
	0.25	13.71	−10.52
	1.00	6.29	−7.95
	2.50	2.44	14.28
NF	0.025	18.42	19.06
	0.25	6.41	−9.20
	1.00	9.60	−2.09
	2.50	2.61	11.83

NFOH hydroxymethylnitrofurazone, *NF* nitrofurazone, *RSD* relative standard deviation

4 Conclusion

The identification of an effective method to quantify new drugs in animal models is a challenge nowadays. In the present work, the development of a bioanalytical method to quantify the new chemical entity, NFOH, in serum using HPLC/UV–Vis provided good selectivity, linearity, accuracy, and precision. It also showed no carryover and matrix effect. The preparation of the sample demonstrated to be simple and suitable for the analysis. Moreover, the proposed method was accessible, fast, and efficient to separate NFOH and NF. This bioanalytical method opens opportunities for quantification of NFOH preparation in serum, to promote NFOH as a new alternative for treating leishmaniasis.

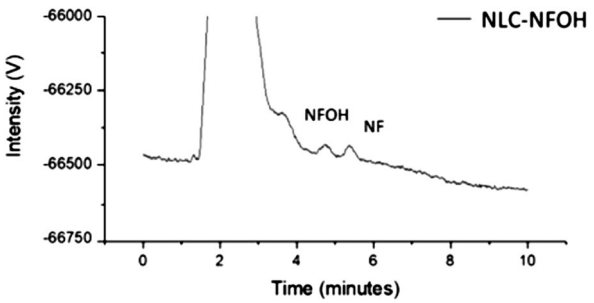

Fig. 4 HPLC Chromatogram of rat serum for quantification of NFOH after oral administration of NLC-NFOH. Chromatographic conditions: mobile phase—acetonitrile:water (20:80); wavelength = 370 nm; volume of injection = 20 µL

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42250-022-00547-6.

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Data availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that that could influence this work.

Ethics approval The Ethics Committee of the Faculty of Medicine of the University of São Paulo approved the animal experiment protocol for this work (protocol n°1155/2019).

References

1. Chung MC, Carvalho Güido RV, Favarato Martinelli T et al (2003) Synthesis and in vitro evaluation of potential anticha-gasic hydroxymethylnitrofurazone (NFOH-121): a new nitro-furazone prodrug. Bioorg Med Chem 11:4779–4783. https://doi.org/10.1016/j.bmc.2003.07.004
2. Monteiro LM, Löbenberg R, Ferreira EI et al (2017) Target-ing Leishmania amazonensis amastigotes through macrophage internalisation of a hydroxymethylnitrofurazone nanostructured polymeric system. Int J Antimicrob Agents 50:88–92. https://doi.org/10.1016/j.ijantimicag.2017.01.033
3. Serafim EOP, de Albuquerque E, Silva AT, de Haro MA et al (2013) Pharmacokinetics of hydroxymethylnitrofurazone, a promising new prodrug for Chagas' disease treatment. Antimi-crob Agents Chemother 57:6106–6109. https://doi.org/10.1128/AAC.02522-12
4. World Health Organization (2020) WHO | Leishmaniasis—global health observatory (GHO) data. https://www.who.int/gho/neglected_diseases/leishmaniasis/en/. Accessed 6 May 2020
5. Chappuis F, Sundar S, Hailu A et al (2007) Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? Nat Rev Microbiol 5:S7–S16. https://doi.org/10.1038/nrmicro1748z
6. Snoussi Y, Sifaoui I, Khalil AM et al (2022) Facile synthesis of silver decorated biochar as a novel and highly active biosourced anti-kinetoplastid agent. Mater Today Commun 32:104126. https://doi.org/10.1016/J.MTCOMM.2022.104126
7. Snoussi Y, Sifaoui I, Garah M el, et al (2022) Green, zero-waste pathway to fabricate supported nanocatalysts and anti-kineto-plastid agents from sugarcane bagasse. https://doi.org/10.26434/CHEMRXIV-2022-712RS
8. Monteiro LM, Löbenberg R, Cotrim PC et al (2017) Buparvaquone nanostructured lipid carrier: development of an affordable deliv-ery system for the treatment of leishmaniases. Biomed Res Int 2017:1–11. https://doi.org/10.1155/2017/9781603
9. Nguyen VH, Thuy VN, Van TV et al (2022) Nanostructured lipid carriers and their potential applications for versatile drug delivery via oral administration. OpenNano. https://doi.org/10.1016/J.ONANO.2022.100064
10. Elmowafy M, Al-Sanea MM (2021) Nanostructured lipid carriers (NLCs) as drug delivery platform: advances in formulation and delivery strategies. Saudi Pharm J 29:999–1012. https://doi.org/10.1016/J.JSPS.2021.07.015
11. Rebouças-Silva J, Tadini MC, Devequi-Nunes D et al (2020) Evaluation of in vitro and in vivo efficacy of a novel amphotericin B-loaded nanostructured lipid carrier in the treatment of *Leish-mania braziliensis* infection. Int J Nanomedicine 15:8659. https://doi.org/10.2147/IJN.S262642
12. Monteiro LM, Löbenberg R, Fotaki N et al (2019) Co-delivery of buparvaquone and polymyxin B in a nanostructured lipid carrier for leishmaniasis treatment. J Glob Antimicrob Resist 18:279–283
13. Das S, Ghosh S, De AK, Bera T (2017) Oral delivery of ursolic acid-loaded nanostructured lipid carrier coated with chitosan oli-gosaccharides: Development, characterization, in vitro and in vivo assessment for the therapy of leishmaniasis. Int J Biol Macromol 102:996–1008. https://doi.org/10.1016/J.IJBIOMAC.2017.04.098
14. Tubino M, Vila MMDC, Palumbo MN (2009) Determination of nitrofurazone in topical pharmaceutical preparations: comparison of the UV–Visible diffuse reflectance versus transmittance versus HPLC methods. J Braz Chem Soc 20:1901–1907. https://doi.org/10.1590/S0103-50532009001000019
15. Wang JR, Zhang LY (2007) Simultaneous determination and iden-tification of furazolidone, furaltadone, nitrofurazone, and nitro-vin in feeds by HPLC and LC-MS. https://doi.org/10.1080/10826070500451962
16. Shen C, Shen B, Liu X, Yuan H (2018) Nanosuspensions based gel as delivery system of nitrofurazone for enhanced dermal bio-availability. J Drug Deliv Sci Technol 43:1–11. https://doi.org/10.1016/j.jddst.2017.09.012
17. Liu X, Shen B, Shen C et al (2018) Nanoparticle-loaded gels for topical delivery of nitrofurazone: effect of particle size on skin permeation and retention. J Drug Deliv Sci Technol 45:367–372. https://doi.org/10.1016/J.JDDST.2018.04.005
18. Shaalan RA, Belal TS (2010) HPLC-DAD stability indicating determination of nitrofurazone and lidocaine hydrochloride in their combined topical dosage form. J Chromatogr Sci 48:647–653. https://doi.org/10.1093/CHROMSCI/48.8.647
19. Monteiro LM, Tavares GD, Ferreira EI et al (2015) Reverse phase high-performance liquid chromatography for quantification of hydroxymethylnitrofurazone in polymeric nanoparticles. Braz J Pharm Sci 51:561–568. https://doi.org/10.1590/S1984-82502015000300008
20. Filho MAFN, Padilha EC, de Campos ML et al (2013) Pharma-cokinetics of hydroxymethylnitrofurazone and its parent drug nitrofurazone in rabbits. Drug Metab Lett 7:58–64. https://doi.org/10.2174/18723128112069990013
21. de Souza A, Yukuyama MN, Barbosa EJ et al (2020) A new medium-throughput screening design approach for the develop-ment of hydroxymethylnitrofurazone (NFOH) nanostructured lipid carrier for treating leishmaniasis. Colloids Surf B Biointer-faces 193:111097. https://doi.org/10.1016/j.colsurfb.2020.111097
22. FDA (2018) Bioanalytical Method Validation Guidance for Indus-try. US Department of Health and Human Services, Food and Drug Administration, p 1–41
23. Ingle RG, Zeng S, Jiang H, Fang WJ (2022) Current developments of bioanalytical sample preparation techniques in pharmaceuti-cals. J Pharm Anal 12:517–529. https://doi.org/10.1016/J.JPHA.2022.03.001
24. Kishikawa N, El-Maghrabey MH, Kuroda N (2019) Chromato-graphic methods and sample pretreatment techniques for alde-hydes determination in biological, food, and environmental

samples. J Pharm Biomed Anal 175:112782. https://doi.org/10.1016/j.jpba.2019.112782
25. Yamashita K, Motohashi M, Yashiki T (1989) Sensitive high-performance liquid chromatographic determination of ionic drugs in biological fluids with short-wavelength ultraviolet detection using column switching combined with ion-pair chromatography: application to basic compounds. J Chromatogr B Biomed Sci Appl 487:357–363. https://doi.org/10.1016/S0378-4347(00)83043-1
26. Pepermans V, Chapel S, Heinisch S, Desmet G (2021) Detailed numerical study of the peak shapes of neutral analytes injected at high solvent strength in short reversed-phase liquid chromatography columns and comparison with experimental observations. J Chromatogr A 1643:462078. https://doi.org/10.1016/j.chroma.2021.462078
27. Fang C-L, Al-Suwayeh SA, Fang J-Y (2013) Nanostructured lipid carriers (NLCs) for drug delivery and targeting. Recent Pat Nanotechnol 7:41–55. https://doi.org/10.2174/187221013804484827