Development of solid drug nanoparticle dispersions for pulmonary delivery of niclosamide and nitazoxanide *via* vibrating mesh nebulisation

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Electronic Supplementary Information

Materials

Nitazoxanide (NTZ) and niclosamide (NCL) were purchased from Biosynth LTD (formerly Carbosynth LTD, Compton, UK). Analytical grade acetone, ethanol and anhydrous dimethyl sulfoxide (DMSO), as well as acetonitrile (HPLC grade), Tween®20, Tween® 80 and formic acid (LC-MS grade) were obtained from Fisher Scientific UK LTD (Loughborough, UK). Pluronic® F127 (F127), sucrose, lactose, poly (vinyl alcohol) (PVA; M_w 9,000-10,000, 80% hydrolysed), Pluronic® F68 (F68), sodium dodecyl sulfate (SDS), hydroxypropyl methylcellulose (HPMC; average M_w 10,000), hydroxypropyl cellulose (HPC; average M_w 100,000), dimethyl sulfoxide-d6 (99.9 atom % D), benzyl methacrylate (96%, contains monomethyl ether hydroquinone), ammonium acetate (HPLC grade), and sodium chloride were purchased from Merck Life Science UK LTD (Gillingham, UK). All materials were used without further purification or preparation.

Methods

Screening of excipient combinations at 70 wt% NTZ, 20 wt% primary excipient, 10 wt% secondary excipient

Excipient stock solutions were prepared at 25 mg mL^{-1} in water. Primary excipient (4 mL), secondary excipient (2 mL) and DI-H_2O (2.25 mL) were added to a 14 mL vial equipped with a magnetic stirrer bar. NTZ (350 mg) was dissolved in anhydrous DMSO (1.75 mL) and rapidly added to the aqueous excipients being stirred at 800 rpm. The subsequent suspension was immediately passed through a spray dryer at a flowrate of 5 mL min^{-1} (Buchi Mini B-290; Aspirator 100%, Nitrogen (cylinder pressure 5 bar) Q-flow gauge 45, Outlet temperature 65 °C). Typical yields for spray dried powders were 35%. The powder was collected and residual water was removed by freeze drying (Virtis Benchtop Pro).

Table S1: Summary of formulations consisting of 70 wt% NTZ that failed the initial screening process due to non-viable dispersed particle properties. The formulations were stabilised using 20 wt% of primary excipient and 10 wt% of secondary excipient, with D_z and PdI determined via DLS analysis of a sample dispersed at 1 mg mL^{-1} in 0.9% w/v saline.

Primary Excipient	**Secondary Excipient**	**D_z (nm)**	**PdI**
PVA	Tween®20	1411	0.724
PVA	Tween®80	3173	-
PVA	SDS	2983	-
F127	Tween®20	1429	0.351
F127	Tween®80	1662	0.632
F127	SDS	1941	0.722
HPMC	Tween®20	1976	0.685
HPMC	Tween®80	2371	0.451
HPMC	SDS	3025	-
F68	Tween®20	2737	-
F68	Tween®80	870	0.219
F68	SDS	1693	0.783
HPC	Tween®20	1570	0.507
HPC	Tween®80	2172	0.682
HPC	SDS	2884	0.020

Method for Nitazoxanide / Pluronic F127 / Tween 20 / Lactose Formulations

All formulations were performed targeting a 5% w/v solids content, 500 mg total solids, and 10 mL total volume. Furthermore, all formulations were performed targeting a 50 wt.% nitazoxanide content whilst varying the targeted composition of pluronic F127, Tween 20, and lactose.

Using the nitazoxanide / Pluronic F127 / Tween 20 / lactose (50/30/10/10) as a specific example, the following method was used:

F127 (3.75 mL; 40 mg mL^{-1} in water), Tween 20 (1.25 mL; 40 mg mL^{-1} in water), lactose (1.25 mL; 40 mg mL^{-1} in water) and water (2.75 mL) were added to a 14 mL vial equipped with a magnetic stirrer bar. Nitazoxanide (250 mg) was dissolved in anhydrous DMSO (1 mL) and rapidly added to the aqueous excipients being stirred at 800 rpm. The subsequent suspension was immediately passed through a spray dryer at a flow rate of 5 mL/minute (Buchi Mini B-290; Aspirator 100%, Nitrogen (cylinder pressure 5 bar), Q-flow gauge 45, outlet temperature 65 °C). The powder was collected, and residual water was removed by freeze-drying (Virtis Benchtop Pro). The spray-dried powder was collected with a yield of 34%.

Table S2: Summary of formulations consisting of 50 wt% NTZ with varying wt% of F127, Tween®20, and lactose in a tertiary stabilised system. D_z and PdI determined via DLS analysis of a sample dispersed at 1 mg mL^{-1} in 0.9% w/v saline.

wt% NTZ	wt% Pluronic F127	wt% Tween®20	wt% lactose	Dz (nm)	PDI
50	30	10	10	742	0.344
50	30	10	10	803	0.413
50	30	0	20	902	0.437
50	25	0	25	852	0.372
50	20	10	20	929	0.488
50	20	0	30	860	0.282
50	15	5	30	1054	0.442
50	10	0	40	1275	0.548
50	10	20	20	1337	0.438
50	10	30	10	1159	0.396
50	0	10	40	1818	0.583

Method for Nitazoxanide / Pluronic F127 / Lactose Formulations

All formulations were performed targeting a 5% w/v solids content, 500 mg total solids, and 10 mL total volume. Furthermore, formulations were performed targeting either a 50 wt.% or 60 wt.% nitazoxanide content whilst varying the targeted composition of pluronic F127 and lactose.

Using the nitazoxanide / Pluronic F127 / lactose (50/30/20) formulation as a specific example, the following method was used:

F127 (3.75 mL; 40 mg mL^{-1} in water), lactose (2.5 mL; 40 mg mL^{-1} in water) and water (2.75 mL) were added to a 14 mL vial equipped with a magnetic stirrer bar. Nitazoxanide (250 mg) was dissolved in anhydrous DMSO (1 mL) and rapidly added to the aqueous excipients being stirred at 800 rpm. The subsequent suspension was immediately passed through a spray dryer at a flow rate of 5 mL/minute (Buchi Mini B-290; Aspirator 100%, Nitrogen (cylinder pressure 5 bar), Q-flow gauge 45, outlet temperature 65 °C). The powder was collected, and residual water was removed by freeze-drying (Virtis Benchtop Pro). The spray-dried powder was collected with a yield of 44%.

Using the nitazoxanide / pluronic F127 / lactose (60/30/10) formulation as another specific example, the following method was used:

F127 (3.75 mL; 40 mg mL^{-1} in water), lactose (1.25 mL; 40 mg mL^{-1} in water) and water (4 mL) were added to a 14 mL vial equipped with a magnetic stirrer bar. Nitazoxanide (300 mg) was dissolved in anhydrous DMSO (1 mL) and rapidly added to the aqueous excipients being stirred at 800 rpm. The subsequent suspension was immediately passed through a spray dryer at a flow rate of 5 mL/minute (Buchi Mini B-290; Aspirator 100%, Nitrogen (cylinder pressure 5 bar), Q-flow gauge 45, outlet temperature 65 °C). The powder was collected, and residual water was removed by freeze-drying (Virtis Benchtop Pro). The spray-dried powder was collected with a yield of 26%.

Method for Nitazoxanide / Pluronic F127 / Sucrose Formulations

All formulations were performed targeting a 5% w/v solids content, 500 mg total solids, and 10 mL total volume. The formulations were performed targeting a 60 wt.% nitazoxanide content whilst varying the targeted composition of pluronic F127 and sucrose.

Using the nitazoxanide / Pluronic F127 / sucrose (60/30/10) formulation as a specific example, the following method was used:

F127 (3.75 mL; 40 mg mL^{-1} in water), sucrose (1.25 mL; 40 mg mL^{-1} in water) and water (4 mL) were added to a 14 mL vial equipped with a magnetic stirrer bar. Nitazoxanide (300 mg) was dissolved in anhydrous DMSO (1 mL) and rapidly added to the aqueous excipients being stirred at 800 rpm. The subsequent suspension was immediately passed through a spray dryer at a flow rate of 5 mL/minute (Buchi Mini B-290; Aspirator 100%, Nitrogen (cylinder pressure 5 bar), Q-flow gauge 45, outlet temperature 65 °C). The powder was collected, and residual water was removed by freeze-drying (Virtis Benchtop Pro). The spray-dried powder was collected with a yield of 25%.

NTZ scale-up sample

F127 (37.5 mL; 40 mg mL^{-1} in water) and sucrose (37.5 mL; 40 mg mL^{-1} in water) were added to a 250 mL sample jar containing DI-H_2O (60 mL). NTZ (4.5 g) was added to a 40 ml sample vial and dissolved in DMSO (15 mL). This solution was heated to 60 °C and allowed to equilibrate to temperature for 10 minutes. The NTZ-DMSO solution was then added to the excipient solution at a flowrate of 5 mL min^{-1} using a peristaltic pump, whilst simultaneously homogenising the excipient solution at 9500 rpm using an IKA Yellowline DI25 basic homogeniser. The subsequent dispersion was homogenised for a further two minutes following complete addition of the drug. The solution was immediately passed through the spray dryer at a flowrate of 5 ml min^{-1} (Buchi Mini B-290; Aspirator 100%, Nitrogen (cylinder pressure 5 bar) Q-flow gauge 45, Outlet temperature 65 °C). Spray dried powder was collected, with a yield of 56%.

DLS characterisation of dispersed SDNs

Spray dried SDN powders for inhalation studies were dispersed in 0.9% w/v saline and analysed with the same instrumentation. Analytical parameters for each dispersant are listed in Table S3. Samples were measured in triplicate (backscattering detector, 25 °C sample temperature), and z-average hydrodynamic diameter (D_z) and polydispersity index (PdI) were used to analyse and compare particle size characteristics. The number of runs per measurement and the degree of light transmission to the detector (attenuator values) were assessed automatically within the software.

Table S3: Measurement parameters used for dynamic light scattering analysis using Zetasizer Advance Ultra device, and ZSXplorer software (Malvern Panalytical, UK)

Particle properties	Refractive Index 1.330, Absorption 0.010
Dispersant	Saline (Viscosity 0.9024 mPa.s^{-1}, Refractive Index 1.330)
Temperature	25 °C
Cell Type	Polystyrene disposable cuvette – 10 mm
Measurement Angle	173° Back Scatter
Number of Measurements	3

High performance Liquid chromatography method for the analysis of NTZ

An Agilent 1200 Series HPLC System equipped with a photodiode array detector was used for the analysis. The separation was achieved under isocratic conditions using an Agilent Poroshell 120 EC-C18, 2.7 µm, 4.6 x 50 mm column and a mobile phase consisting of 20 mM ammonium acetate aqueous buffer solution and acetonitrile (ammonium acetate$_{(aq.)}$/acetonitrile, 70/30). The column oven was set to 30 °C. A flow rate of 0.5 mL min^{-1} and an injection volume of 2 µL was used. Detection was carried out using a wavelength of 425 ± 4 nm. All calibration standards and formulation samples were prepared in H_2O/acetonitrile/DMSO (60/39/1), filtered through a 0.22 µm PTFE filter and immediately analysed following preparation. The NTZ calibration curve was generated between 10 µg mL^{-1} and 50 µg mL^{-1} to give a linear correlation with a correlation coefficient of 0.9998

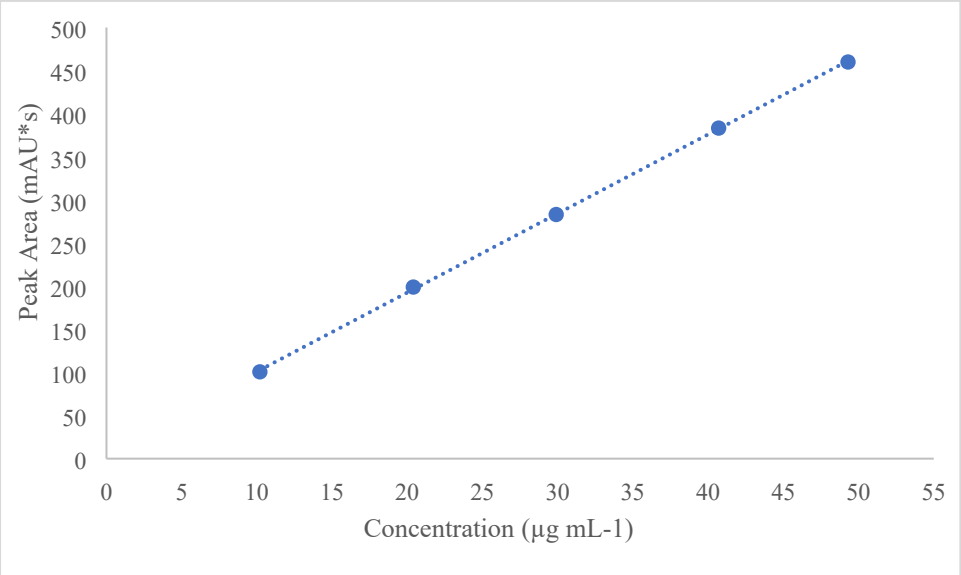

Figure S1: The NTZ calibration curve generated between 10 µg mL^{-1} and 50 µg mL^{-1}, showing the line equation and R^2 value.

Chemical Composition Analysis by ^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were obtained using a Bruker Avance spectrometer operating at 400 MHz. Chemical shifts (δ) are reported in parts per million (ppm). The drug composition of the formulations was determined using a known concentration of benzyl methacrylate (BzMA) as an internal standard. Comparison of integrations between the resonances of the internal standard and known resonances of the drug enabled calculation of the moles of drug and therefore the mass of drug within the sample. NTZ formulations were run in DMSO-d_6 with a 10 mg mL^{-1} concentration of BzMA (Figure S2). Whereas NCL formulations were run in DMF-d_7 with a 5 mg mL^{-1} concentration of BzMA.

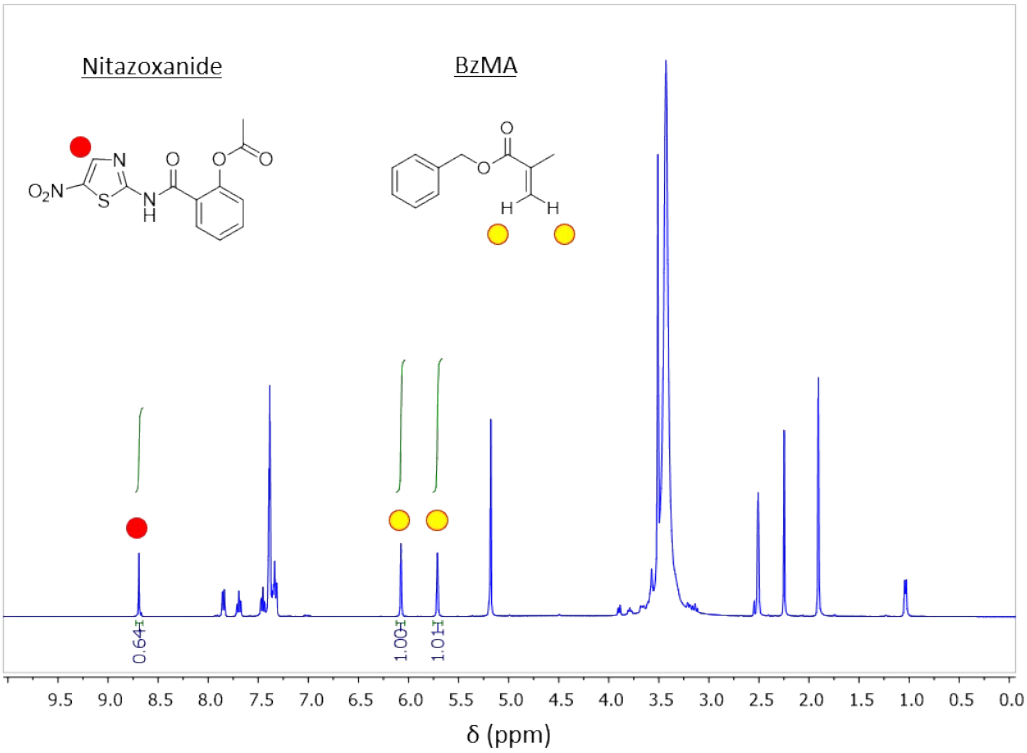

Figure S2: ^{1}H-NMR (DMSO-d_6) analysis of NTZ/F127/sucrose (60/20/20 wt%) large-scale formulation. Sample mass of 20.0 mg. Benzyl methacrylate (BzMA, 10 mg mL^{-1}) as internal standard. Moles of BzMA within the NMR sample = 5.675 x10^{-5} mol. Therefore, 3.632 x10^{-5} mol and 11.2 mg of NTZ within the NMR sample. Calculated drug composition of 56 wt%.

High performance Liquid chromatography method for the analysis of NCL

HPLC analysis was conducted using an Agilent 1660 Infinity II instrument, fitted with an Agilent Poroshell EC-C18 column. The column dimensions were 4.6 mm by 100 mm and the pore size within was 2.7 µm.

A multistep gradient method was employed, with mobile phase A consisting of H_2O with 0.1% formic acid, and mobile phase B consisting of acetonitrile with 0.1% formic acid. The total run time was 7 minutes at a flow rate of 1 mL min^{-1}, with the column oven set to 40 °C. The gradient steps were as shown in table S4

Table S4. Gradient method for HPLC analysis of NCL:

Time (min)	Flow rate (mL min^{-1})	Mobile phase A (%)	Mobile phase B (%)
0.0	1	95	5
0.5	1	50	50
5.5	1	99	1
6.0	1	99	1
6.01	1	5	95
7.0	1	5	95

A calibration curve was generated by serial dilution and covered a concentration range of 312 -40,000 $ng.mL^{-1}$. The NCL had a retention time of approximately 5 minutes, an example chromatogram is shown (figure S3).

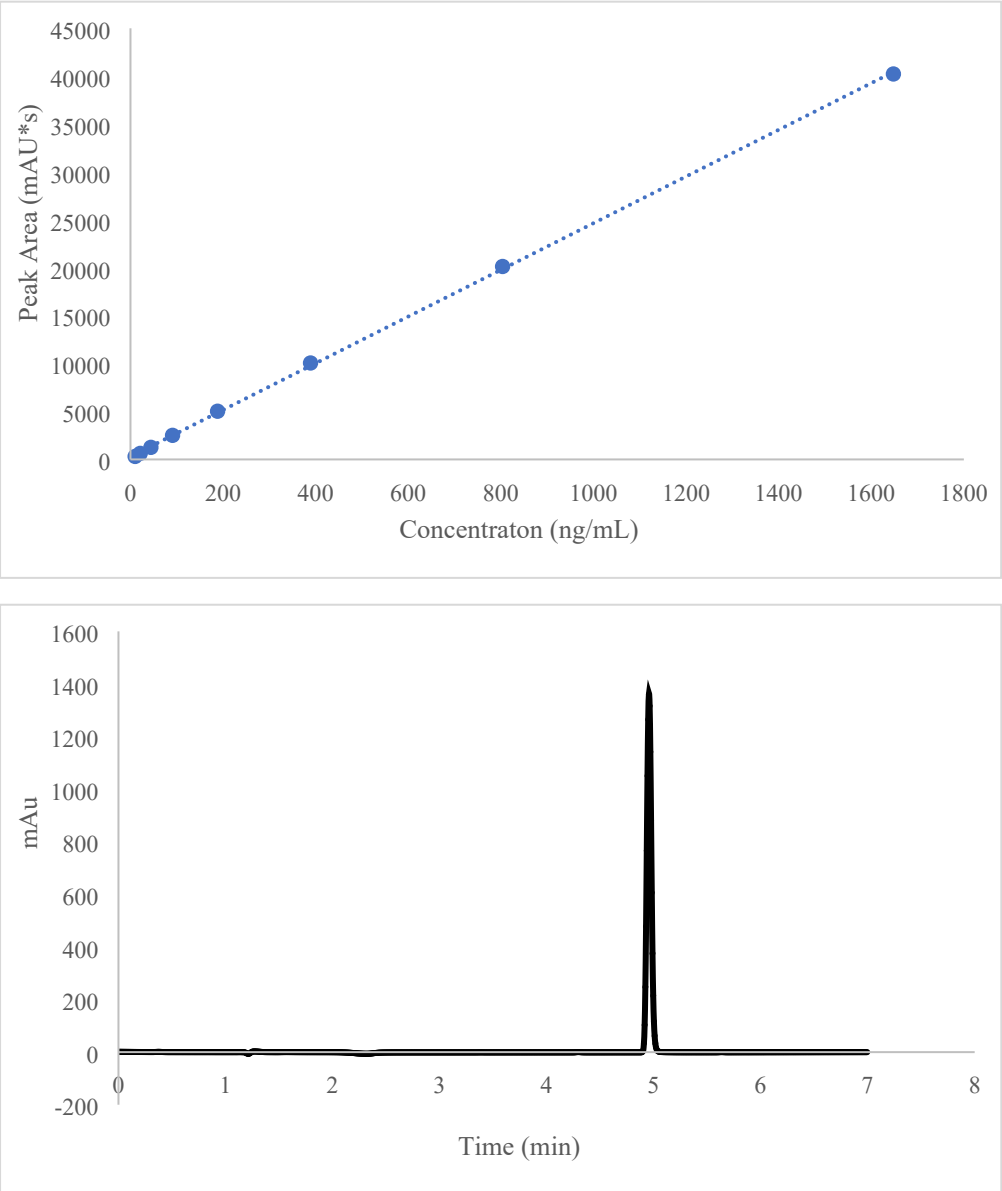

Figure S3. 8-point calibration curve for NCL covering a range of 312-40,000 $ng.mL^{-1}$ (top) and an example chromatogram of NCL (bottom).

Droplet size analysis – cascade impaction

Droplet size (mass median aerodynamic diameter - MMAD & geometric standard deviation - GSD) results for the metabolite of NTZ - tizoxanide (TIZ). Samples were run at 5 & 10 mg mL^{-1} with separate nebulisers used for each run. The NGI was operated at 15 litres per minute, and at 5 degrees Celsius. NOTE: TIZ is the active metabolite of NTZ that is readily formed even under mild conditions. These MMAD values are a by-product of the NTZ testing in Table 4.

Table S5: Results of droplet size analysis for TIZ using NGI cascade impactor. Results are presented as MMAD and GSD, plus calculation of Fine Particle Fraction percentage (FPF) at ≤5µm, 2-5 µm and ≤2 µm cut-offs.

	MMAD (µm)	GSD	FPF (%) ≤5 µm	FPF (%) 2-5 µm	FPF (%) ≤2 µm	LOR (mL/min)
TIZ						
5 mg mL^{-1}	4.496	2.044	56.192	47.352	8.840	0.41
	4.355	2.078	59.538	52.426	7.112	0.28
	4.767	1.967	52.612	46.695	5.917	0.37
10 mg mL^{-1}	4.090	1.928	63.529	55.910	7.619	0.31
	4.305	1.895	60.450	54.489	5.961	0.29
	4.000	1.897	64.672	55.942	8.730	0.29

Scanning electron microscopy imaging of unformulated API powders, spray dried SDN powders and dispersions

Scanning electron microscopy (SEM) images were obtained using a Tescan FIB SEM S8000G equipped with cryo stage (Tescan-UK Ltd., Cambridge, UK). Powder samples were prepared by mounting on stubs and sealing edges with electro dag, followed by coating in chromium using a Quorum S150T ES sputter coater (Quorum, Laughton, East Sussex, UK) to improve electrical conductivity. Samples were imaged using an acceleration voltage of 1 kV and a current of 30 mA.

Figure S4: Scanning electron microscopy analysis of unformulated bulk NTZ powder

Nebulisation studies

Nebulisation studies were carried out using an Aerogen Solo vibrating mesh nebuliser (Aerogen, Galway, Ireland) in combination with the Aerogen ProX controller (Aerogen, Galway, Ireland). SDNs were dispersed in 0.9% w/v saline and loaded into the the nebuliser. Suspensions were delivered over a period of 25 minutes and collected in 50 mL centrifuged tubes sealed with Parafilm™. The collected suspension was weighed to assess recovery and delivery rate and characterised by DLS.

In vitro determination of inhaled niclosamide after nebulisation

The following was carried out by Aerogen (Galway, Ireland). To simulate inhaled dose in a clinical setting, a Maquet-Servo-i® mechanical ventilator (Getinge, Derby, UK) was used to generate three different breathing patterns in the assessment of the inhaled lung dose of two SDN dispersion concentrations. Adapted from the method described by Naughton *et al*., NCL was dispersed in a solution of albuterol which was used to determine droplet destination and collected on capture filters placed at the entrance of a test lung. Filters were washed and the recovered albuterol was quantified by UV spectrophotometry. Mass balance recovery from filters was confirmed to be 100 ± 5 %.

Cascade impaction of aerosolised niclosamide and nitazoxanide

The following was carried out by Aerogen (Galway, Ireland). SDN dispersions were separated by cascade impaction using a Next Generation Impactor (NGI, Copley Scientific, Nottingham, UK) at 15 L min^{-1}. SDN powder was dispersed at 5 and 10 mg mL^{-1} in 0.9% w/v saline. 1 mL of dispersion was loaded into an Aerogen Solo VMN (Aerogen, Galway, Ireland) which was attached to the NGI. At the end of nebulisation, the equipment was disassembled, and the separated material was recovered after rinsing with 0.9% w/v saline. Separated material was returned to the Centre of Excellence for Long-acting Therapeutics, University of Liverpool and quantified by HPLC