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Quantitation of tizoxanide in multiple matrices to support cell culture, animal and human research

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ABSTRACT

Currently nitazoxanide is being assessed as a candidate therapeutic for SARS-CoV-2. Nitazoxanide is rapidly broken down to its active metabolite tizoxanide upon administration. Unlike many other candidates being investigated, tizoxanide plasma concentrations achieve antiviral levels after administration of the approved dose, although higher doses are expected to be needed to maintain these concentrations across the dosing interval in the majority of patients. Here an LC-MS/MS assay is described that has been validated in accordance with Food and Drug Administration (FDA) guidelines. Fundamental parameters have been evaluated, and these included accuracy, precision and sensitivity. The assay was validated for human plasma, mouse plasma and Dulbecco's Modified Eagles Medium (DMEM) containing varying concentrations of Foetal Bovine Serum (FBS). Matrix effects are a well-documented source of concern for chromatographic analysis, with the potential to impact various stages of the analytical process, including suppression or enhancement of ionisation. Herein a validated LC-MS/MS analytical method is presented capable of quantifying tizoxanide in multiple matrices with minimal impact of matrix effects. The validated assay presented here was linear from 15.6 ng/mL to 1000 ng/mL. The presented assay here has applications in both pre-clinical and clinical research and may be used to facilitate further investigations into the application of nitazoxanide against SARS-CoV-2.

1. Introduction

Nitazoxanide (NTZ) is a thiazolide anti-protozoal drug, approved to treat diarrhoea caused by the parasites *Cryptosporidium* and *Giardia* in children and adults [1,2], and is administered orally as a suspension or tablet twice daily with food [2]. Additional efficacy has been demonstrated both *in vitro* and *in vivo* against a wide range of parasites, bacteria, fungi and viruses [3-9]. This broad-spectrum of activity is in part attributed to NTZs rapid transformation into its deacetylated active metabolite tizoxanide (TIZ) upon administration [4]. NTZ has previously been considered for drug repurposing to treat respiratory viral infections [9], including Middle Eastern respiratory syndrome (MERS) [10], with clinical trials having assessed its suitability to treat acute influenza [11,12].

NTZ has been assessed as a candidate therapeutic for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with 25 trials listed on clinicaltrials.gov [13]. *In vitro* evaluation of the activity of NTZ against SARS-CoV-2 in Vero E6 cells generated an EC_{90} estimated at 4.65 μM [14,15]. Subsequently, TIZ was additionally confirmed to have antiviral activity with an EC_{90} 3.16 μM under similar assay conditions [16]. Unlike many other candidates which have been investigated, TIZ plasma concentration achieve antiviral levels after administration of the approved dose [14], although higher doses are expected to be needed to maintain these concentrations across the dosing interval in the majority of patients [17]. Several mechanisms of action for NTZ against SARS-CoV-2 have been proposed and are summarised by Lokhande *et al.* [18]. NTZ has previously been demonstrated to inhibit production of pro-inflammatory cytokines and interleukin-6 in peripheral blood mononuclear cells *in vitro* [19], and reduce interleukin-6 plasma levels in mice [20]. It has been hypothesised that this may assist host anti-

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inflammatory responses during SARS-CoV-2 disease progression and thus lessen the SARS-CoV-2 cytokine storm [9,18].

Research into the suitability of NTZ as a treatment for SARS-CoV-2 includes dose optimisation, tolerability and drug-interaction studies [17,21]. One uncertainty relating to the putative utility of NTZ is that TIZ is highly protein bound in the plasma of humans [22]. However, whether this binding is high affinity / low capacity or low affinity / high capacity is currently unknown and could have a profound impact upon the probability of success [23,24]. Methods for quantification of concentrations of TIZ in a variety of matrices are required to better understand the exposure–response relationship, *in vitro, in vivo* and in humans.

Currently few validated liquid chromatography tandem mass spectrometry (LC-MS/MS) assays are available to quantify NTZ and TIZ in relevant biological matrices [25-27]. Previous studies include a validated LC-MS method for quantifying NTZ and TIZ in mouse plasma, lung, BAL cells, liver, spleen kidney and heart with a limit of quantification (LOQ) of 0.97 ng/mL [26]. As well as a LC-MS/MS methods validated in goat faeces with a limit of detection of 5 ng/g [27]. High performance liquid chromatography (HPLC) method has also been validated for NTZ and TIZ in rat plasma and human plasma, urine and breast milk [28,29].

Here an LC-MS/MS assay is described that has been validated in accordance with Food and Drug Administration (FDA) guidelines [30]. Fundamental parameters have been evaluated, and these included accuracy, precision and sensitivity. Other validation criteria also included linearity, recovery and repeatability of TIZ within selected matrices. The assay was validated for human plasma, mouse plasma and Dulbecco's Modified Eagles Medium (DMEM) containing varying concentrations of Foetal Bovine Serum (FBS). Matrix effects are a well-documented source of concern for chromatographic analysis, with the potential to impact various stages of the analytical process [31], including suppression or enhancement of ionisation [30]. Therefore, a validated LC-MS/MS analytical method is presented capable of quantifying TIZ in multiple matrices with minimal impact of matrix effects.

2. Methods and materials

2.1. Materials

TIZ and the internal standard (IS) TIZ-D4 were purchased from Toronto Research Chemicals inc (Toronto, Canada). Drug free mouse plasma and human plasma with lithium heparin were purchased from VWR International (PA, USA). LCMS grade acetonitrile (ACN) was purchased from Fisher Scientific (MA, USA). All other consumables were purchased from Merck Life Science UK LTD (Gillingham, UK) and were of LC-MS grade.

2.2. Tuning for tizoxanide and internal standard

A stock solution of 1 mg/mL of TIZ or TIZ-D4 was prepared in ACN. From this stock a solution of 10 ng/mL was prepared in H2O: methanol (50:50) for tuning. Detection was performed using a SCIEX 6500 + Qtrap (SCIEX MA, USA) operating in negative mode. TIZ or TIZ-D4 was infused at 10 μL/min in order to optimise compound-specific parameters (declustering potential, collision energy and collision exit potential) and source specific parameters (curtain gas, ionisation voltage, temperature, nebuliser gas and auxiliary gas).

2.3. Chromatographic separation

TIZ was resolved using a kinetex C18 column (2.7 μm, 2.1 × 100 mm Phenomenex CA, USA) using a 5 μL injection. The following multistep gradient was used with H_2O + 0.1% formic acid (mobile phase A) and ACN + 0.1% formic acid (mobile phase B) at a flow rate of 0.4 mL/min: the initial conditions were 95% A and 5% B and held for 1 min. Mobile phase B was rapidly increased to 80% over 0.5 mins. The gradient was then slowly increased to 95% B till 4 mins. This was held for 0.5 mins when the initial conditions were returned and held for 0.5mins.

2.4. Extraction from mouse plasma, human plasma and foetal bovine serum

The assay described here was developed to quantify TIZ from *in vitro* (DMEM plus 50%, 25%, 12%, 10%, 5% and 2% FBS or 50% and 10% human plasma) and *in vivo* (mouse plasma and human plasma) matrices to support SARS-CoV-2 preclinical research. 100 μL of standard quality control (QC) sample or untreated matrix was pipetted into 7 mL glass tubes. 500 μL of ACN containing IS (20 ng/mL) was added to each tube and thoroughly vortexed. Following vortexing, the samples were centrifuged at 3500 rpm for 5mins at 4 °C. 500 μL of supernatant was then transferred to fresh 7 mL tubes and the samples were evaporated to dryness under a gentle stream of nitrogen. All samples were reconstituted in 100 μL of reconstitution buffer (30% H_2O, 49% ACN and 21% DMF). 50 μL was the transferred to chromatography vials and analysed.

2.5. Calibration curve

A nine-point calibration curve ranging from 15.6 ng/mL to 1000 ng/mL, was prepared by spiking untreated matrix with TIZ followed by serial dilution in untreated matrix. A blank untreated matrix sample was also included. TIZ calibrator and QC samples were prepared from the same stock solution of 1 mg/mL of TIZ prepared in ACN. Linearity was assessed over 3 independent runs. Acceptance criteria were as follows; deviation of interpolated standards from stated concentrations were set at 15%, excluding the lower limit of quantification (LLOQ) where deviation was set at no more than 20%. Acceptable R^2 was set at greater than 0.99.

2.6. Recovery

Recovery was assessed at 3 concentrations (40 ng/mL, 400 ng/mL and 800 ng/mL) covering the dynamic range of the assay. The peak area of extracted samples was compared to post-extracted blank matrix spiked with 40 ng/mL, 400 ng/mL and 800 ng/mL.

2.7. Selectivity and specificity

Specificity was determined by comparing the signal produced by an extracted blank to the peak produced by the lower limit of quantification (LLOQ). FDA guidelines require the signal of endogenous interference be no more than 20% of the LLOQ.

2.8. Carryover

Carryover was determined by determining the signal produced by a blank following an injection of the upper limit of quantification (ULOQ). FDA guidelines require the signal of endogenous interference be no more than 20% of the LLOQ.

2.9. Accuracy and precision

Accuracy and precision were determined for intra- and inter-assay variability. The degree of variation was calculated by determining the % of the interpolated concentration of QCs to the expected concentration. Accuracy (% variability of accuracy = interpolated value/expected value*100) and precision (%variation of precision = standard deviation/mean value*100) were determined at 4 concentrations (15.6 ng/mL, 40 ng/mL, 400 ng/mL and 800 ng/mL) in triplicate. Acceptable variation for accuracy and precision was set at 15% and at 20% for the lower concentrations.

Table 1
Shows the optimised parameters used to detect TIZ and TIZ-D4 (IS).

Parent (*m/z*)	Product (*m/z*)	RT (Min)	DP (Volts)	EP (Volts)	CE (Volts)	CXP (Volts)
Tizoxanide	*216.8	2.45	−160	−10	−18	−23
263.9	**113.9	2.45	−160	−10	−28	−11
Tizoxanide-D4 (IS) 267.9	221.0	2.45	−160	−10	−20	−19

Global Parameters	**Curtain Gas**	**Collision Gas**	**Voltage (V)**	**Temperature (°C)**	**Gas 1**	**Gas 2**
	50	Medium	−4500	450	50	50

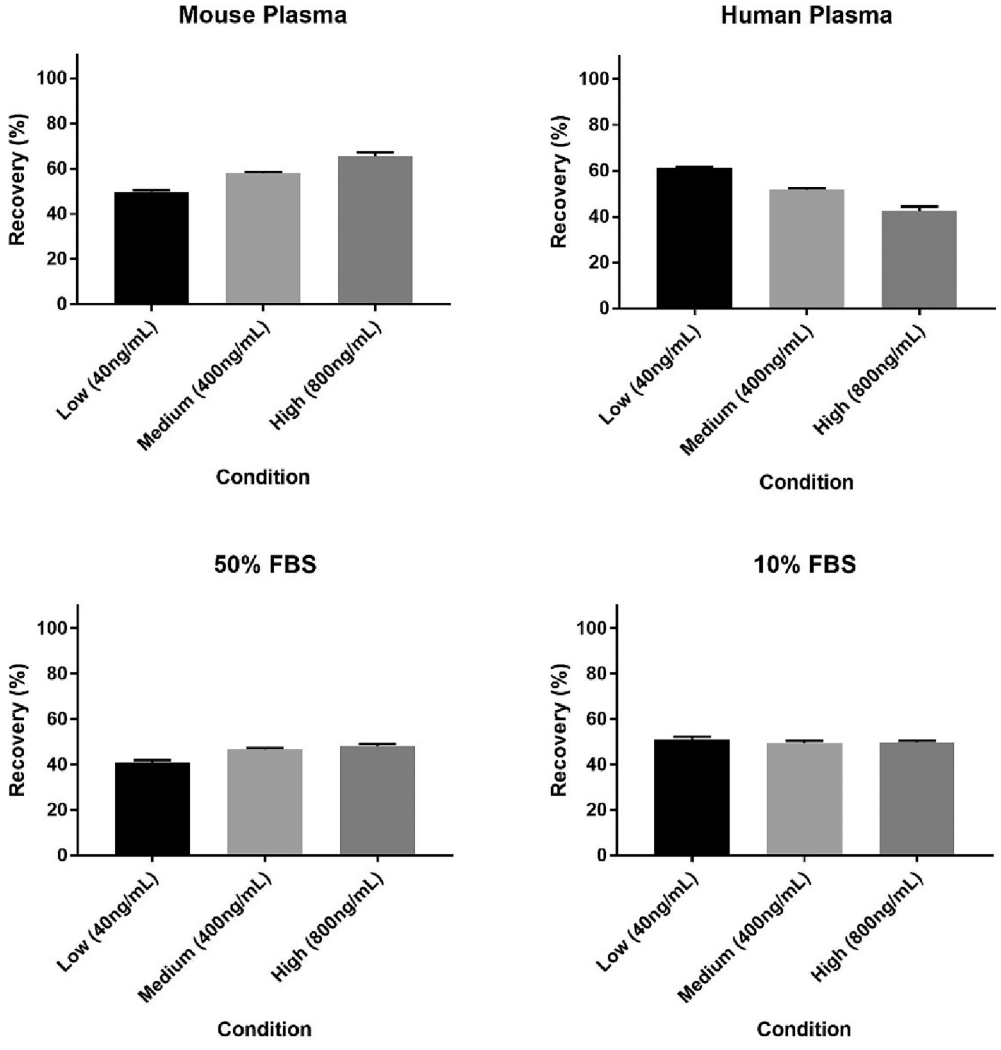

Fig. 1. Shows the recovery of TIZ in mouse plasma, human plasma, 50% FBS and 10% FBS (±SD).

2.10. Tizoxanide concentrations quantified from preclinical study samples

The degree of TIZ protein binding to FBS and Human plasma in DMEM was determined using rapid equilibrium dialysis (RED). Three hundred microliters of DMEM or DMEM supplemented with either 2%, 5%, 10%, 12%, 25%, 50% FBS or 10%, 50%, 100% Human plasma all containing 10 μM TIZ (<1% DMSO) were placed into separate dialysis chambers with an 8 KDa MWCO (ThermoFisher). Five hundred microliters of PBS, pH 7.4, was placed into each corresponding buffer chamber of the RED insert. Subsequently, the plate was sealed with Parafilm and incubated at 37 °C, 250 rpm on an orbital shaker (Grant Instruments, UK) for 4 h. Following incubation 200 μL of both the dialysis and buffer chambers were sampled and quantified using the TIZ LC-MS/MS method outlined above. The fraction of unbound TIZ (*fu*) was calculated through division of the TIZ concentration in the buffer chamber dialysate over the TIZ concentration remaining in the sample chamber.

3. Results

3.1. Recovery of tizoxanide

TIZ and IS were detected using the optimised analyte specific and global parameters found in Table 1. The mean recovery was 57.7%

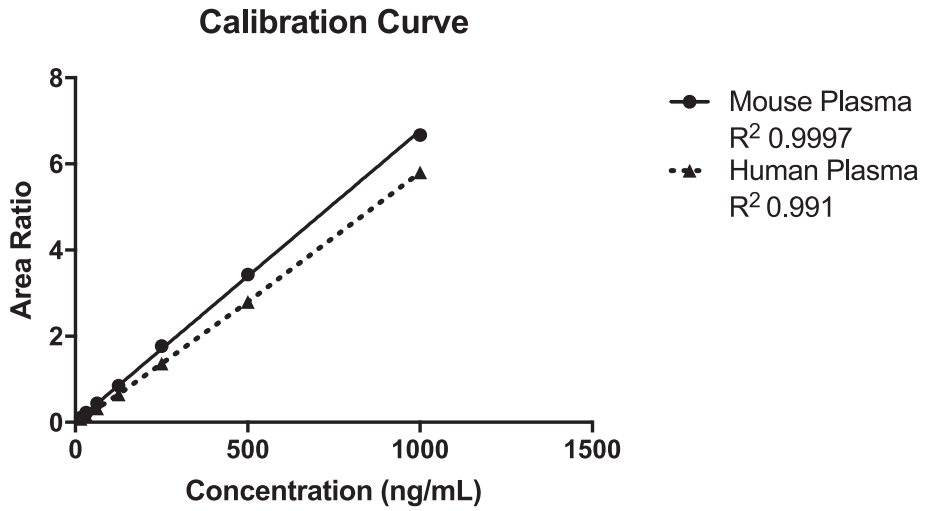

Fig. 2. Shows example standard curves produced in mouse and human plasma. Also shown is the R^2 of the regression.

Table 2
Shows the endogenous signal from an extracted blank compared to the LLOQ of TIZ and the % of the LLOQ. FDA guidelines require the signal produced by an extracted blank be no more than 20% of the LLOQ. Mouse plasma and FBS are from pooled sources and human plasma is from a single source.

Matrix	Blank Signal	LLOQ Peak Area	% of LLOQ	Blank following ULOQ	% Carryover
Mouse plasma	221	29787.3	0.7	474	1.6
Human plasma	1291	89042.2	1.4	13100.2	14.7
50% Human plasma	3134	101150.3	3.1	13192.3	13.0
10% Human plasma	3071	76959.5	4.0	12240.6	15.9
50% FBS	1697	59069.4	2.9	11284.6	19.1
25% FBS	2683	61746.9	4.3	11950.2	19.4
12% FBS	2287	64418.4	3.6	10691.9	16.6
10% FBS	1641	85137.6	1.9	13799.9	16.2
5% FBS	4869	102319.1	4.8	16,354	16.0
2% FBS	6571	104790.3	6.3	18519.7	17.7

±7.93 and 51.8% ±9.50 for mouse plasma and human plasma, respectively. Recovery from DMEM with varying percentages of serum was 45.2% ±3.84 (50% FBS), 48.1% ±4.72 (25% FBS), 50.3% ±6.54 (12% FBS), 50.1% ±0.63 (10% FBS), 42.1% ±4.20 (5% FBS), 42.8% ±4.50 (2% FBS), 50.5% ±9.80 (0% FBS), 51.0% ±2.30 (50% human plasma) and 40.5% ±4.68 (10% human plasma). While recovery varied between each matrix, mean recovery within each matrix demonstrated high repeatability (example recoveries are shown in Fig. 1).

3.2. Linearity

Extracted calibrators demonstrated strong linearity (mouse plasma $R^2 = 0.9997$, human plasma $R^2 = 0.9991$, 50% human plasma $R^2 = 0.9983$, 10% human plasma $R^2 = 0.9992$, 50% FBS $R^2 = 0.9970$, 25% FBS $R^2 = 0.9992$, 12% FBS $R^2 = 0.9996$, 10% FBS $R^2 = 0.9992$, 5% FBS $R^2 = 0.9995$ and 2% FBS $R^2 = 0.9995$) meeting all acceptance criteria (Fig. 2). The calibration curve was fit to the data using a linear equation with sample weighting of 1/X.

3.3. Selectivity

Interference from endogenous compounds of a matrix is a well-documented confounding effect in bioanalysis. The Peak area produced by the LLOQ was compared to detectable signal at the retention time of TIZ (2.46 mins) and expressed as a percentage of the peak area of the LLOQ (Table 2). Representative chromatograms are shown in Fig. 3.

3.4. Carryover

Carryover was assessed by comparing the peak area of a blank following an injection of the ULOQ to the peak area of the LLOQ. Table 2 shows the % carryover observed in ech matrix. Although carryover was higher in FBS matrices, all carryover signals were below 20% of the LLOQ.

3.5. Accuracy and precision

The assay described here was fully validated using mouse plasma. The variation in accuracy and precision was determined within each assay (inter-assay) and across 3 replicates of the assay (intra-assay). Variance in accuracy and precision was determined by comparing the interpolated values of extracted LLOQ and QCs to the known values. Inter assay fell below 15% for accuracy (range −13.5% −2.2%) and precision (range 0.9–5.4%) at all concentrations across each replicate run. Mean intra assay variance in accuracy and precision was −3.1% and 3.6% respectively. FDA guidelines require part validation for minor changes to the assay (e.g., change of matrix). The remaining matrices were assessed for inter assay variation [30]. The intra-assay % error in accuracy was below 15% at all levels in human plasma (range −3.3 – 8.0) and FBS (range −1.4 – 10.8). Intra-assay error of precision also fell below 15% in human plasma (range 1.8–7.0) and FBS (range 1.1 – 7.0, Table 3) (see Table 4).

3.6. Tizoxanide concentrations quantified from preclinical study samples

The results in Fig. 4 show that TIZ is highly bound to FBS in supplemented DMEM growth media. The degree to which TIZ is bound to FBS appears largely proportional to the percentage supplementation. TIZ was shown to be unbound (*fu* 0.98) in DMEM alone. A 55% reduction in the TIZ free fraction was observed when co-incubated with 2% FBS (*fu* 0.44). The free fraction declined to 0.03 when co-incubated with 50% FBS. A recent review of preprints and papers investigating the anti-SARS-CoV-2 activity of small molecule drugs revealed most studies reported the use of 2–12% serum supplemented media. The majority of these, >85%, reported use of either 2% or 10% serum for efficacy studies [23]. Fig. 4B shows that the free fraction of TIZ is significantly lower when supplemented with 10% (P = 0.0007, two-tailed *t*-test) and 50%

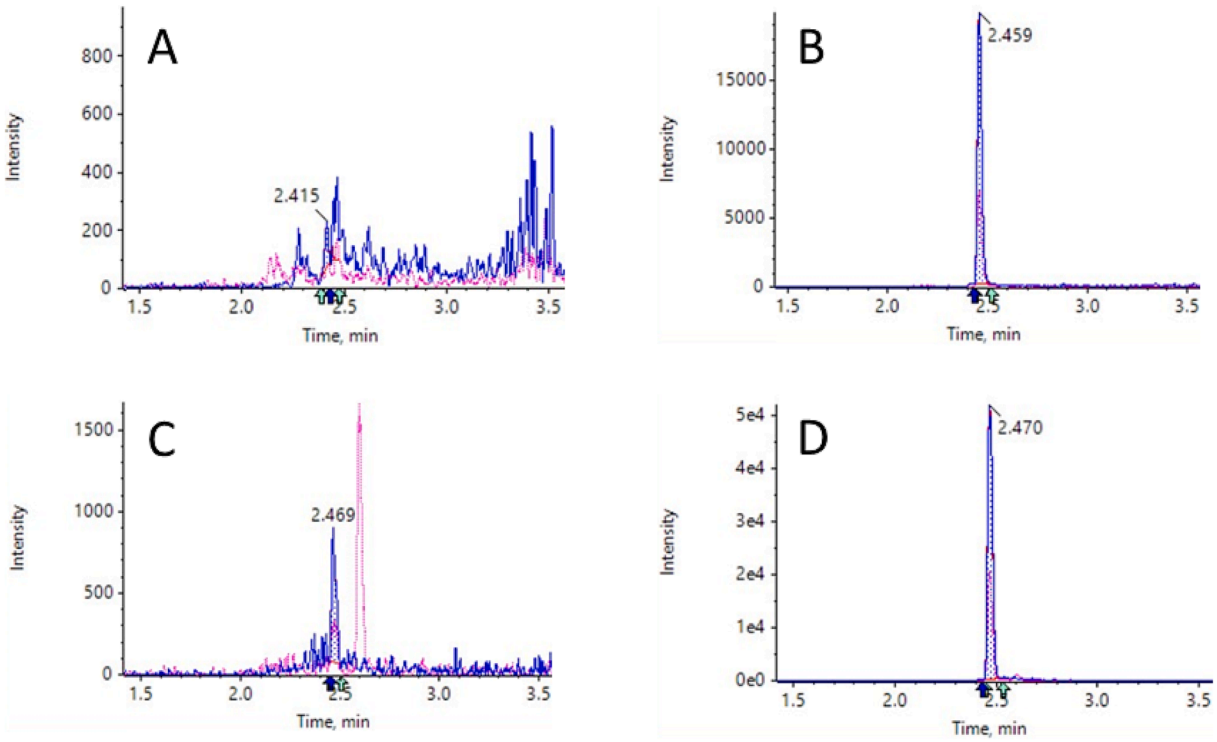

Fig. 3. Shows representative chromatograms of (A) extracted blank mouse plasma, (B) the extracted LLOQ (15.6 ng/mL) in mouse plasma, (C) extracted blank human plasma and (D) the extracted LLOQ (15.6 ng/mL) in human plasma. Chromatograms in blue and red show the signal produced by the daughter fragments of TIZ 216.8 and 113.9 respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
shows the validation of TIZ extracted from mouse plasma. The data shows the average quantitated sample at 3 levels, the % deviation in accuracy and the % deviation in precision. Acceptance criteria is variation no more than 15%, excluding the lower concentration where deviation may be no more than 20%.

		Intra-day			Inter-day		
		Average ± SD (ng/mL)	Variance of accuracy (%)	Variance of precision (%)	Average ± SD (ng/mL)	Variance of accuracy (%)	Variance of precision (%)
Assay 1	15.6 ng/mL	14.9 ± 0.61	−5.0	4.1	15.3 ± 0.41	−2.0	2.7
	40 ng/mL	38.5 ± 1.22	−3.8	3.2	37.0 ± 2.14	−7.4	5.8
	400 ng/mL	408.8 ± 7.81	2.2	1.9	397.9 ± 9.72	−0.5	2.4
	800 ng/mL	798.7 ± 13.9	−0.2	1.7	789.5 ± 19.36	−1.3	2.5
Assay 2	15.6 ng/mL	15.5 ± 2.30	−0.4	14.8			
	40 ng/mL	34.6 ± 1.87	−13.5	5.4			
	400 ng/mL	390.0 ± 11.29	−2.5	2.9			
	800 ng/mL	767.3 ± 38.88	−4.1	5.1			
Assay 3	15.6 ng/mL	15.5 ± 0.48	−0.5	3.1			
	40 ng/mL	38.1 ± 0.33	−4.9	0.9			
	400 ng/mL	395.0 ± 6.23	1.6	1.6			
	800 ng/mL	802.6 ± 20.40	0.3	2.5			

($P = 0.02$, two-tailed *t*-test) Human plasma in DMEM compared with the corresponding FBS supplemented DMEM.

4. Discussion

The anti-SARS-CoV-2 activity of NTZ is being investigated *in vitro*, *in vivo*, and in clinical trials. Unlike many candidates being explored for direct repurposing as SARS-CoV-2 antivirals, the pharmacokinetics of NTZ do suggest that antiviral concentrations can be achieved in humans when benchmarked against targets derived from *in vitro* studies. However, uncertainty about high protein binding of TIZ in plasma, the specific mechanism of action, and the appropriate dose necessitates a careful understanding of the exposure–response relationship. A recent multicentre, randomised, double-blind, placebo-controlled trial, in adult patients presenting up to 3 days after onset of symptoms and with PCR-confirmed SARS-CoV-2 infections indicated some antiviral activity at

Table 4
Shows the part validation of each of the investigated matrices. The data shows the average quantitated sample at 3 levels, the % deviation in accuracy and the % deviation in precision. Acceptance criteria is variation no more than 15%, excluding the lower concentration where deviation may be no more than 20%.

		Average ± SD (ng/mL)	Intra-day	
			Variance of accuracy (%)	Variance of precision (%)
Human Plasma	40 ng/mL	40.4 ± 1.04	1.0	2.6
	400 ng/mL	394.2 ± 22.70	−1.4	7.0
	800 ng/mL	840.3 ± 36.74	5.0	4.4
Human Plasma 50%	40 ng/mL	40.2 ± 1.02	0.6	2.5
	400 ng/mL	387.0 ± 10.87	−3.3	2.8
	800 ng/mL	784.6 ± 16.78	−1.9	2.1
Human Plasma 10%	40 ng/mL	43.2 ± 0.78	8.0	1.8
	400 ng/mL	403.1 ± 8.51	0.8	2.1
	800 ng/mL	840.8 ± 15.95	5.1	1.9
FBS 50%	40 ng/mL	40.0 ± 1.72	−0.1	4.3
	400 ng/mL	405.7 ± 9.86	1.4	2.4
	800 ng/mL	833.1 ± 15.28	4.1	1.8
FBS 25%	40 ng/mL	40.8 ± 0.55	2.1	1.4
	400 ng/mL	400.8 ± 8.35	0.2	2.1
	800 ng/mL	788.9 ± 34.32	−1.4	4.4
FBS 12%	40 ng/mL	40.4 ± 1.12	1.1	2.8
	400 ng/mL	407.5 ± 14.52	1.9	3.6
	800 ng/mL	831.3 ± 14.10	3.9	1.7
FBS 10%	40 ng/mL	40.4 ± 1.04	1.0	2.6
	400 ng/mL	394.2 ± 27.70	−1.4	7.0
	800 ng/mL	840.3 ± 36.74	5.0	4.4
FBS 5%	40 ng/mL	41.4 ± 0.47	3.4	1.1
	400 ng/mL	413.6 ± 22.76	3.4	5.5
	800 ng/mL	846.3 ± 36.53	5.8	4.3
FBS 2%	40 ng/mL	42.5 ± 1.01	6.3	2.4
	400 ng/mL	434.2 ± 14.70	8.5	3.4
	800 ng/mL	886.0 ± 28.48	10.8	3.2

500 mg three times a day [32]. However, much more data are required to support the candidacy of NTZ (including at higher doses), and studies should incorporate an understanding of the pharmacokinetics during SARS-CoV-2 infection.

A greater understanding of the pharmacokinetic-pharmacodynamic relationship requires validated LC-MS/MS methods with utility in relevant biological matrices. The selection of matrices within this study included human and mouse plasma and DMEM plus 50%-2% FBS or 50%-10% human plasma. The methods presented here enable quantification of TIZ in a range of matrices chosen for their relevance to clinical, *in vitro* and *in vivo* investigations of NTZ as a SARS-CoV-2 therapeutic. The assay sensitivity enables quantification of TIZ at concentrations relevant to that observed in *in vitro* assays, as demonstrated in the presentation of the results from an *in vitro* study, and that observed in human plasma samples taken from other studies [29]. This offers a novel methodology distinct from what is currently in the literature, where human plasma concentrations of TIZ are only quantifiable at much lower sensitivity through HPLC methods [25-29].

Although human and mouse plasma have some homogeneity, species differences can lead to changes in assay behaviour. As demonstrated by protein binding being commonly lower in preclinical species than humans [33]. Furthermore, differences in *in vitro* methods can often result in differential percentages of FBS being used to supplement DMEM in different studies. Given that previously outlined changes in matrix have the potential to impact assay performance, and that this problem could be further compounded by the high protein binding observed for TIZ, >99.9% in plasma [2], a thorough investigation of multiple matrices was warranted. Small differences in recovery of TIZ within different matrices were evident, but all recovery percentages were seen to be repeatable for each of the matrices (deviation < 15%). Furthermore, the endogenous signal from each of the matrices was seen to be less than 7% of the LLOQ, falling well within the 20% acceptance criteria set out in FDA guidance [30]. Thus, the change in matrix is unlikely to meaningfully impact assay performance.

The decision to focus on TIZ exclusively was driven by the observation that NTZ is rapidly converted to TIZ, and that previous studies have not detected NTZ in plasma, the main matrix of interest [34]. The assay was able to quantify TIZ within multiple matrices, generating highly repeatable, precise and accurate results, indicating a tolerance to the negative impact of matrix effects. The described assay will be further developed to include the major metabolite of TIZ, tizoxanide glucuronide. In summary, a highly sensitive TIZ LC-MS/MS assay is presented, which fully meets FDA bioanalytical method development guidelines.

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CRediT authorship contribution statement

Megan Neary: Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Usman Arshad:** Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Lee Tatham:** Data curation, Investigation, Writing – review & editing. **Henry Pertinez:** Data curation, Formal analysis, Writing – review & editing. **Helen Box:** Visualization, Writing – review & editing. **Rajith K. R. Rajoli:** Data curation, Writing – review & editing. **Anthony Valentijn:** Investigation, Formal analysis, Writing – review & editing. **Joanne Sharp:** Conceptualization, Project administration, Writing – review & editing. **Steve P. Rannard:** Conceptualization, Funding acquisition, Writing – review & editing. **Giancarlo A. Biagini:** Conceptualization, Funding acquisition, Writing – review & editing. **Paul Curley:** Conceptualization, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Andrew Owen:** Conceptualization, Funding acquisition, Resources, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Andrew Owen reports financial support was provided by UK Research and Innovation. Andrew Owen reports financial support was provided

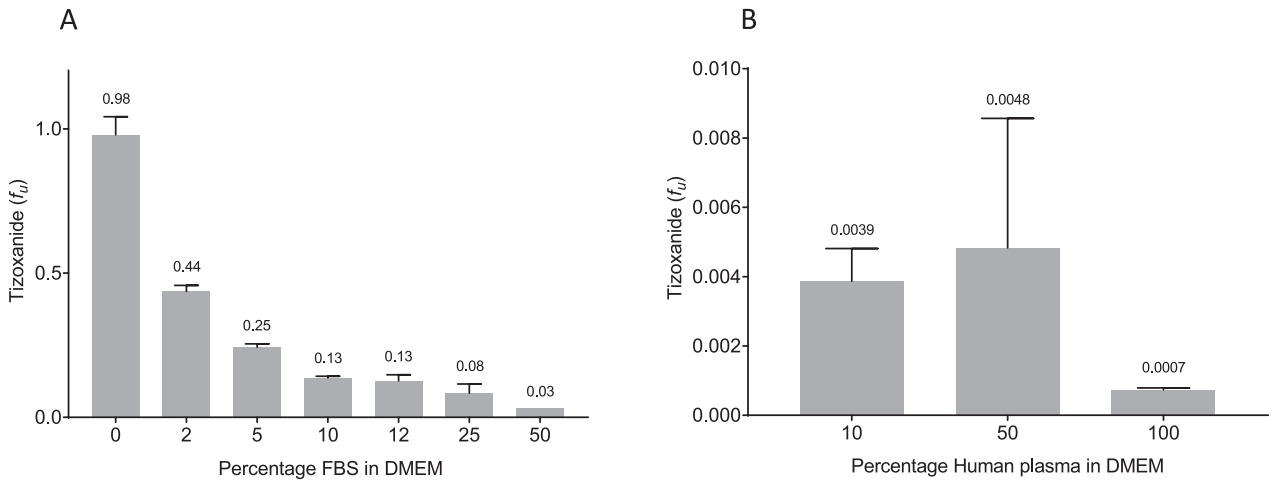

Fig. 4. TIZ fraction unbound (*fu*) in DMEM containing different percentages of (A) FBS or (B) Human plasma following 4 h incubation of the RED plate at 37 °C, 250 rpm. TIZ was quantified using the outlined LC-MS/MS methods. Data are means ± SD (n = 3).

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Data availability

Data will be made available on request.

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