SUPPLEMENTARY MATERIAL:

Title:

A Green Analytical Method for Identification and Quantification of Carcinogenic Impurity N-Nitroso-Desformyl Riociguat in Riociguat Formulations by UHPLC-MS/MS

Authorship:

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Supplemental Materials:

From the research results of Analytical Method for Identification and Quantification of Genotoxic Impurity N-Nitroso-Desformyl Riociguat by Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS) in Riociguat Drug Product, herewith provided the reference Table(s) few reference Chromatograms, and a few supplementary material.

Discussion:

Quantitative Environmental Impact Comparison

When compared to traditional nitrosamine analysis techniques, the developed UHPLC-MS/MS technology significantly reduced environmental effect. Below detailed comprehensive quantitative evaluations revealed practical sustainability gains in a number of areas. (Refer Table S6 for Reduction Comparison)

a) Solvent Consumption Reduction:

The method consumed approximately 15 mL total organic solvent per sample (10 mL MTBE for extraction, 5 mL methanol for reconstitution and mobile phase contribution), representing a 65% reduction compared to traditional GC-MS methods that typically require 40-50 mL per sample for extraction and derivatization procedures. Over 100 samples analyzed annually in a typical quality control laboratory, this translated to a reduction of 2.5-3.5 L of organic solvent waste, equivalent to approximately 6-8 kg CO_2 emission savings based on solvent lifecycle analysis.

b) Sample Preparation Efficiency:

The liquid-liquid extraction procedure required 35 minutes per sample (including vortexing, centrifugation, extraction, evaporation, and reconstitution), compared to 90-120 minutes for solid-phase extraction methods commonly employed for nitrosamine analysis. This 60-70% time reduction directly decreased energy consumption from laboratory equipment operation. Specifically, centrifugation (6 minutes at 200 W) and nitrogen evaporation (15 minutes at 150 W) consumed approximately 0.058 kWh per sample, compared to 0.180-0.220 kWh for SPE-based approaches requiring vacuum manifolds and extended drying steps.

c) Analysis Time Optimization:

The 15-minute chromatographic runtime enabled processing of 4 samples per hour, achieving superior throughput compared to 25-30 minute(s) methods reported for nitrosamine analysis in pharmaceuticals. When accounting for sample preparation time, the total analytical cycle (preparation + analysis) was completed in 50 minutes per sample, compared to 2.5-3.5 hours for conventional approaches. This efficiency improvement reduced instrument operation time by approximately 75%, translating to proportional decreases in energy consumption (UHPLC-MS/MS power draw: 1.2 kW) and extended instrument lifetime through reduced wear.

d) Waste Minimization:

The method generated approximately 25 mL total waste per sample (15 mL organic waste, 10 mL aqueous waste), classified as non-halogenated organic waste suitable for standard solvent recycling programs. Conventional approaches generated 60-80 mL mixed waste per sample, often including chlorinated solvents requiring specialized hazardous waste disposal. The 65-70% waste reduction resulted in estimated disposal cost savings of $15-20 per sample and reduced environmental burden associated with waste incineration or specialized treatment.

e) Carbon Footprint Assessment:

A comprehensive lifecycle assessment considering solvent production, energy consumption, waste disposal, and consumable materials estimated the carbon footprint at approximately 0.85 kg CO_2-equivalent per sample. This represented a 55-60% reduction compared to conventional methods (1.9-2.1 kg CO_2-eq/sample), primarily attributable to reduced solvent consumption and shorter analysis time. When extrapolated to routine pharmaceutical quality control applications (estimated 500-1000 samples annually), the method contributed to annual carbon footprint reductions of 525-1,150 kg CO_2-equivalent per laboratory.

Table S1: Optimized Method conditions of the Chromatography hyphenated Mass Spectrometry (LC-MS/MS) with a Gradient Program

Optimized Chromatographic Conditions:		
Injection Volume	:	10 µL
Flow Rate	:	0.5 mL/min. (Flow gradient)
Sampler Temperature	:	25°C
Column Temperature	:	35°C
Run Time	:	15.0 min
Optimized Mass Spectrometric Conditions:		
Ion source type	:	Ion Focus Electrospray Ionization source (HESI)
Interface Heater	:	On
Interface Temperature	:	250°C
Desolvation Temperature	:	444°C
DL Temperature	:	200°C
Nebulizing Gas Flow	:	3.00 L/min
Heating Gas Flow	:	3.00 L/min
Heat Block	:	200°C
Drying Gas Flow	:	3.00 L/min
CID Gas	:	200 kpa
Chromatographic peak width	:	06 sec
Start and End Time	:	0.0 min and 15.0 min

Gradient Program			
Time (min)	**Flow rate (mL/min)**	**Mobile Phase A (%v/v)**	**Mobile Phase B (%v/v)**
0.00	0.5	98	2.0
4.00	0.5	98	2.0
4.50	0.5	60	40.0
8.00	0.5	60	40.0
8.50	0.5	10	90.0
12.00	0.5	10	90.0
12.10	0.5	98	2.0
15.00	0.5	98	2.0

Acquisition Mode	:	Multiple Reaction Monitoring (MRM) as per below conditions

Compound	Polarity	Precursor (m/z)	Product (m/z)	Dwell Time	Q1 Pre Bias	CE	Q3 Pre Bias	Q1 Resolution	Q3 Resolution	Interface Volt. (kv)	Focus Volt. (kv)
NNDFR (Quantifier)	positive	394.20	364.15	100.00	-40.0	-10.0	-26.0	unit	unit	1.00kV	1.00kV
NNDFR (Qualifier)	positive	394.20	255.15	100.00	-40.0	-24.0	-28.0	unit	unit	1.00kV	1.00kV

Table S2: Fragmentation profile of N-Nitroso-Desformyl Riociguat (NNDFR) under positive ESI conditions

Species	m/z (Obs./ Theor.)	Proposed Structure / Key Functional Groups	Molecular Formula	Mass (Da)	Fragmentation Mechanism	Notes / Diagnostic Value
Parent $[M+H]^+$	452.16 (Theor.)	Protonated NNDFR intact molecule	$C_{20}H_{18}FN_9O_3$	452.1589	Ionization of intact molecule	Full precursor, often unstable under source conditions
Precursor Ion (in-source)	394.20 (Obs.)	Loss of methyl carbamate group (–59 Da)	$C_{18}H_{16}FN_8O^+$	394.1509	In-source fragmentation prior to CID	Used as primary precursor in MRM
Fragment 1 (Quantifier)	364.15 (Obs.)	Loss of nitroso group (–30 Da) from precursor	$C_{18}H_{16}FN_7^+$	364.1529	NO group cleavage, diagnostic for nitrosamines	Monitored as quantifier transition
Fragment 2 (Qualifier)	255.15 (Obs.)	Fluorobenzyl-pyrazolopyridine core with partial pyrimidine	$C_{13}H_{12}FN_4^+$	255.1046	Stepwise loss of nitroso + amino moieties, retention of aromatic scaffold	Monitored as qualifier transition
Fragment 3 (Theoretical)	227.11	Fluorobenzyl-pyrazolopyridine core	$C_{11}H_{10}FN_4^+$	227.0890	CN group loss from Fragment 2	Supportive, theoretical
Fragment 4 (Theoretical)	199.06	Fluorobenzyl-pyrazolopyridine base	$C_9H_7FN_4^+$	199.0733	Pyrimidine ring loss from Fragment 3	Supportive, theoretical
Fragment 5 (Theoretical)	171.04	Fluorobenzyl-pyrazole fragment	$C_7H_5FN_4^+$	171.0577	Pyridine ring loss from Fragment 4	Supportive, theoretical

Species	m/z (Obs./ Theor.)	Proposed Structure / Key Functional Groups	Molecular Formula	Mass (Da)	Fragmentation Mechanism	Notes / Diagnostic Value
Fragment 6 (Theoretical)	109.02	Fluorobenzyl cation	$C_7H_6F^+$	109.0392	Pyrazole ring loss, fluorobenzyl retained	Characteristic terminal fragment

Table S3: Regression results obtained from LOQ level to 200% level solutions

Linearity Levels	N-Nitroso-Desformyl Riociguat Impurity	
	Conc. in (µg/mL)	Avg. Area
LOQ Level	0.00123	660457.5
50% Level	0.00615	2916898
100% Level	0.01230	6490238
125% Level	0.01537	7384412
150% Level	0.01845	9019023
200% Level	0.02459	11947314.5
Slope	**484356327.862**	
%Y-Intercept	**99660.0**	
Correlation Coefficient (R^2)	**1.00**	

Table S4: Evaluation & Inference on Ruggedness and Filter Compatibility

Parameter	Result	Acceptance Criteria
Blank Interference	ND	No interference or < sensitivity solution response
Sensitivity Solution S/N Ratio	152	≥ 10
System Suitability (%RSD)	0.7	≤ 15.0
Cumulative System Suitability (%RSD)	1.2	≤ 20.0 cumulative
%RSD for Spiked Content	0.4	≤ 15.0
Cumulative %RSD for Spiked Content	0.8	≤ 20.0
Filter Compatibility (%Difference)	1.9	≤ ±25.0

Table S5: Evaluation & Inference on Robustness (Low & High Flow Rate and Low & High Column Temp.)

Parameter	Result	Acceptance Criteria
Blank Interference (Low & High Flow Rate and Low & High Column Temp.)	Not Detected	No interference or < sensitivity solution response
Sensitivity Solution S/N Ratio (Low & High Flow Rate and Low & High Column Temp.)	41–955	≥ 10
System Suitability (Low & High Flow Rate and Low & High Column Temp.) (%RSD)	1.9–5.4	≤ 15.0
Cumulative System Suitability (Low & High Flow Rate and Low & High Column Temp.) (%RSD)	1.8–6.5	≤ 20.0 cumulative
%RSD for Spiked Content (Low & High Flow Rate and Low & High Column Temp.)	0.8–6.2	≤ 15.0
%Difference (Low & High Flow Rate and Low & High Column Temp.)	-12.5 to -6.2	≤ ±25.0

Table S6: Quantitative Environmental Impact Comparison

Parameter	This Method	Conventional Methods	Improvement
Organic solvent per sample	15 mL	40-50 mL	65% reduction
Sample prep time	35 min	90-120 min	60-70% reduction
Total analysis time	50 min	150-210 min	67-76% reduction
Energy consumption per sample	0.060 kWh	0.180-0.220 kWh	67-73% reduction
Waste generated per sample	25 mL	60-80 mL	58-69% reduction
Carbon footprint per sample	0.85 kg CO_2-eq	1.9-2.1 kg CO_2-eq	55-60% reduction
Samples per 8-hour shift	9.6	2.7	256% increase
Annual CO_2 savings (500 samples)	--	--	525-625 kg

Figures:

Fig. S1: Blank solution Chromatogram using MRM mode under conditions described in Sections 2.7 and 2.8, confirming absence of interference at the NNDFR retention time.

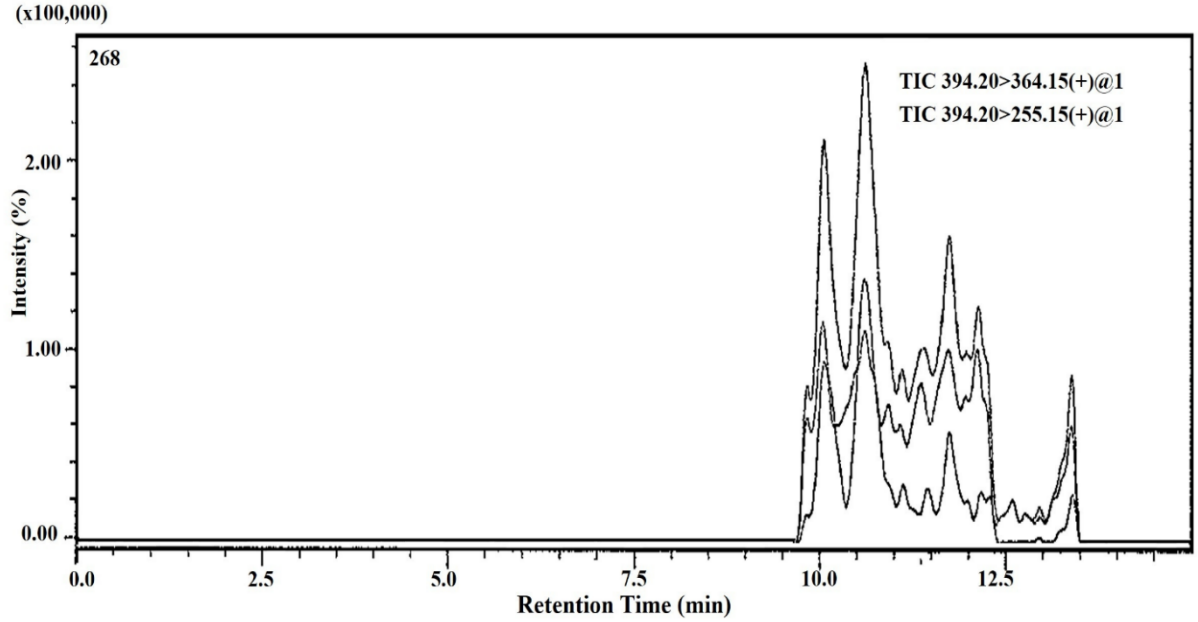

Fig. S2: Quantified Chromatogram of Placebo Solution extract prepared according to Section 2.3, showing no interfering peaks at the NNDFR retention time, supporting method specificity

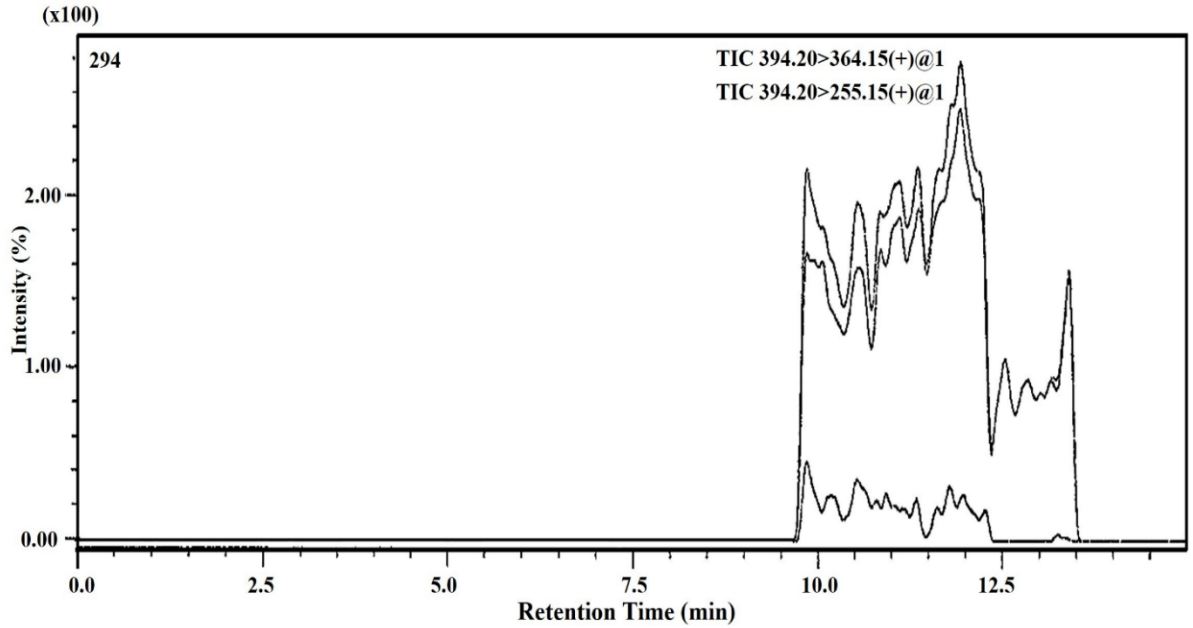

Fig. S3: Representative UHPLC–MS/MS Quantified Chromatogram of NNDFR at the LOD Level (~0.40 ppm)

Note: The displayed trace was obtained by averaging replicate injections (n = 3) to enhance visualization of signal quality and baseline stability. Quantitative determination of LOD was performed on raw unprocessed chromatograms, with an observed signal-to-noise ratio of approximately 3, consistent with ICH criteria.

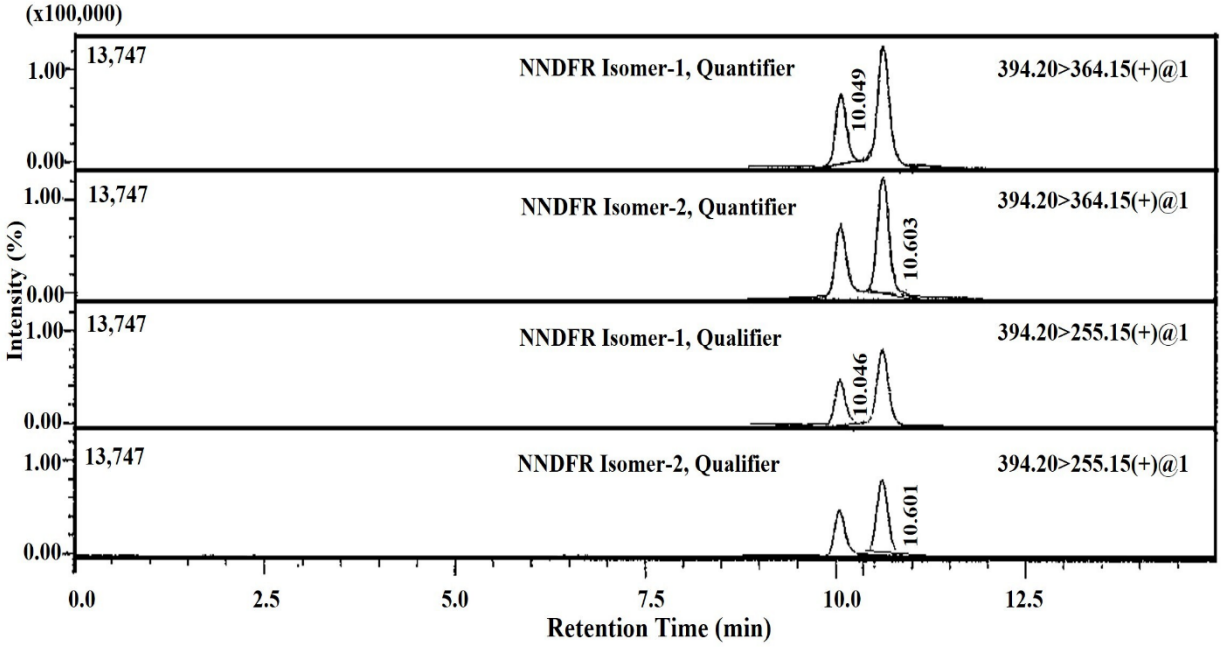

Fig. S4: Representative UHPLC–MS/MS Quantified Chromatogram of NNDFR at the LOQ Level (~1.22 ppm)