Application note | 003910

Pharma

Liquid chromatography – high-resolution mass spectrometry method for determination of NDSRI in six beta-blocker drug substances

Authors

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Keywords

USP, LC-HRMS, beta-blocker, API (active pharmaceutical ingredient), Vanquish Flex UHPLC, Orbitrap Exploris 120 mass spectrometer, NDSRI, Chromeleon CDS, Mass Frontier software

Application benefits

- A single high-resolution-based method to determine the NDSRI level in a beta-blocker drug substance with applicability to six different beta-blocker drug substances, namely atenolol, bisoprolol fumarate, carvedilol, labetalol HCl, metoprolol fumarate, and propranolol HCl.
- A robust gradient method with significant chromatographic separation between the NDSRI and the corresponding drug substance.
- Better retentivity and chromatographic resolution between NDSRI and the drug substance with comparatively shorter runtime.

Goal

Development of a highly accurate and reliable LC-HRMS-based versatile methodology that could be suitable to analyze an NDSRI in a beta-blocker drug substance, which will be applicable for six different drug substances and their related NDSRIs.

Introduction

Beta-blockers, also known as β-blockers, are a class of medications used to manage arrhythmia and to protect the heart from a second heart attack. They are also widely used to treat high blood pressure. Some of them block activation of all types of β-adrenergic receptors, and others are selective for one of the three known types of beta receptors, designated β_1, β_2, β_3 receptors.

NDSRIs[1], also known as nitroso drug substance related impurities, belong to a class of nitrosamine impurities that have been identified in many drug products and could be present in active pharmaceutical ingredients (APIs). These impurities share structural similarity to the API (having the API or API fragment in the chemical structure) and are therefore unique to each API.

NDSRIs generally form in the drug product through nitrosation of APIs (or API fragments) that have secondary or tertiary amines when exposed to nitrosating agents such as residual nitrites in excipients used to formulate the drug product. The presence of nitrosamine impurities[2] (including NDSRIs) at significant levels in drug products is a point of concern globally. There has been a history of drug recalls due to unacceptable levels of nitrosamine impurities in many drug products.

NDSRIs often lack carcinogenicity and mutagenicity study data (typically from animal studies) from which an acceptable intake (AI) limit can be determined. Therefore, multiple regulatory guidance documents are available today to determine the AI for NDSRIs.[1] The US FDA[1] launched a guidance document in August 2023 that provides a recommended methodology for the AI limit determination that uses structural features of NDSRIs to generate a predicted carcinogenic potency categorization and corresponding recommended AI limit that manufacturers and applicants can apply, in the absence of other FDA recommended AI limits, in their evaluations of approved and marketed drug products as well as products in development or under review by the FDA.

Similarly, the EMEA[3] has updated the guidance documents by including Appendix 1, 2, and 3 to share significant recommendations on acceptable intake of NDSRIs and the Carcinogenic Potency Categorization Approach (CPCA) for *N*-nitrosamines to determine the AI value for the NDSRIs.

A major attribute in nitrosamine or NDSRI testing is data accuracy with respect to the analytical technique chosen for the quantitative experiments. Out of multiple techniques available today, a liquid chromatography coupled to high-resolution mass spectrometry approach is the one that provides confidence in data accuracy but also addresses the need of higher selectivity. With respect to mass accuracy in the Thermo Scientific™ Orbitrap Exploris™ 120 high-resolution mass spectrometer, external calibration achieves <3 ppm RMS drift over 24 hours; internal lock mass calibration achieves <1 ppm RMS drift over 24 hours; the Thermo Scientific™ EASY-IC™ ion source achieves <1 ppm RMS drift for at least 5 days. In addition, the One-Point Mass Self-Calibration achieves <3 ppm RMS drift over at least 4 weeks. This exceptional mass accuracy ensures optimal confidence in the analytical results. This helps in excluding isobaric interferences that may contribute to the NDSRI content and may lead to false positive determination of nitrosamines in drug substances or drug products.

In this application development, six beta-blocker drug substances were selected with corresponding NDSRIs. Figure 1 shows the NDSRIs selected in this study. The AI limit for each NDSRI was selected based upon CPCA, and liquid chromatography coupled to high-resolution mass spectrometry was chosen as the analytical technique for accurate and reliable determination of an NDSRI in its respective drug substance. The major guidelines followed, but not limited to, to carry out this application development were those of the US FDA[4] and EMEA[5].

Pertinently, the instrument configuration of an Orbitrap Exploris 120 mass spectrometer connected to a Thermo Scientific™ Vanquish™ Flex UHPLC system as the front end stands to be a suitable configuration for the highly accurate and reliable analysis of NDSRIs in six different beta-blocker drug substances. The method has been evaluated upon various validation parameters and has been found to be satisfactory in terms of meeting the most widely pursued acceptance criteria.

This is a versatile method that is applicable to analyze an NDSRI in a drug substance, and it can be applied to analyze any of the six NDSRIs in their respective drug substances. From data acquisition through reporting, Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) meets all compliance requirements while ensuring robust data integrity and security. Its comprehensive user-management workflows and complete instrument and data audit trails provide full transparency and control.

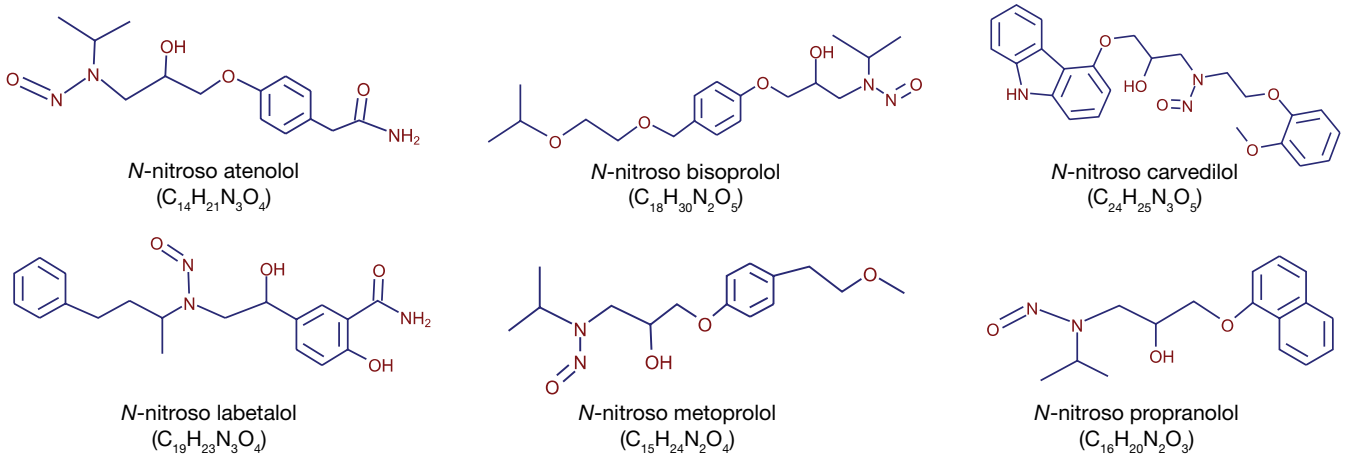


Figure 1. Structures of NDSRIs related to the six selected beta-blocker drug substances.

Experimental

Instrumentation

- Vanquish Flex UHPLC system consisting of:
  - Thermo Scientific™ System Base Vanquish™ Horizon/Flex (P/N VF-S01-A-02)
  - Thermo Scientific™ Vanquish™ Binary Pump F (P/N VF-P10-A)
  - Thermo Scientific™ Vanquish™ Split Sampler FT (P/N VF-A10-A)
  - Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A)
  - Thermo Scientific™ Vanquish™ Diode Array Detector FG (P/N VF-D11-A-01) with 10 mm, SST 13 µL flow cell (P/N 6083.0510)
- Orbitrap Exploris 120 high-resolution mass spectrometer (P/N BRE725531)
- PEAK Scientific™ Genius™ XE nitrogen generator (P/N 3300252)
- SCAT™ HPLC supply and waste set – safety caps with air valves for reagent bottles (P/N 307447)

Software

Data acquisition, data processing, and reporting were performed using Chromeleon CDS, version 7.3.2. Thermo Scientific™ Mass Frontier™ 8.0 SR2 Software was used for spectral interpretation.

Reagents and consumables

- Reference standards from USP
  - *N*-nitroso-atenolol (NNAT)
  - *N*-nitroso-bisoprolol (NNBI)
  - *N*-nitroso-carvedilol (NNCA)
  - *N*-nitroso-labetalol (NNLA)
  - *N*-nitroso-metoprolol (NNME)
  - *N*-nitroso-propranolol (NNPR)
  - Atenolol
  - Bisoprolol fumarate
  - Carvedilol
  - Labetalol HCl
  - Metoprolol fumarate
  - Propranolol HCl
- Fisher Chemical™ Formic acid, Optima™ LC/MS grade (Fisher Scientific P/N A117-50 or equivalent)
- Fisher Chemical™ Methanol, Optima™ LC/MS grade (Fisher Scientific P/N A456-4 or equivalent)
- Fisher Chemical™ Water, Optima™ LC/MS grade (Fisher Scientific P/N AAB-W6-4 or equivalent)
- Invitrogen™ 2 mL microcentrifuge tubes (P/N AM12475)
- Thermo Scientific™ Hypersil GOLD™ C18 Selectivity column, 3 × 100 mm, 3 µm (P/N 25003-103030)
- Thermo Scientific™ Nunc™ 15 mL extraction/conical sterile polypropylene centrifuge tubes (P/N 339650)
- Thermo Scientific™ Nunc™ 50 mL extraction/conical sterile polypropylene centrifuge tubes (P/N 339652)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps (P/N CHSC9-30UBS)
- Thermo Scientific™ SureSTART™ 2 mL Polypropylene Screw Top Microvials 0.4 mL (P/N 6ESV9-04PP)
- Thermo Scientific™ Titan3™ 0.2 µm PVDF syringe filters (P/N 42213-PV)
- Thermo Scientific™ National Target All-Plastic Disposable Syringes 1 mL (P/N S7510-1)

Solutions and sample preparation

Diluent solution and blank solution preparation

Diluent solution was prepared by mixing 50 mL of methanol and 50 mL of water and was used as diluent solution and blank solution.

Standard stock solution preparation

Standard stock solution of 100 µg/mL solution of the respective NDSRI impurity was prepared in methanol and for all further dilutions, diluent solution was used.

Standard solution preparation at specification level with respect to drug substance

The specification limit for each NDSRI, with respect to the related drug substance according to AI and MDD, was calculated as per CPCA category and is found in Table 1.

Standard solution preparation, at specification level, for each NDSRI was done using the diluent solution.

Preparation of LOD, LOQ, and linearity standard solutions

LOD and LOQ standard solutions were prepared in diluent solution as per Table 2. Linearity standards were prepared at eight different concentration levels in increasing order, including 10% and 350% as the lowest and highest mentioned in Table 2. The other six intermediate linearity standards were prepared at 50%, 100%, 150%, 200%, 250%, and 300% concentration levels.

Drug substance sample preparation

The required amount of the drug substance was weighed and dissolved in diluent solution to prepare non-spiked sample or in neat standard solution to prepare spiked sample as per Table 3.

Further, for atenolol, metoprolol fumarate, bisoprolol fumarate and propranolol HCl, the samples were vortexed until complete solubility.

For carvedilol, the sample was sonicated for 5 minutes and vortexed until dissolve completely.

For labetalol HCl, the sample was sonicated for 5 minutes and vortexed for 2 minutes. Then the entire content was transferred into 2 mL micro centrifuge tubes and centrifuged at a speed of 12,000 rpm and at 5 °C for 15 minutes.

Samples were filtered through 0.22 µm PVDF filters and ≤200 µL of the filtered solution was transferred into 0.4 mL polypropylene autosampler vials.

Table 1. Representation of calculation and selection of specification limit as per CPCA and other significant concentrations.

Calculation of specification limit for NDSRIs							
Sr. no.	Drug substances	Name of NDSRI	NDSRI abbreviations for this study	CPCA category	AI (ng/day)	*MDD (mg/day)	Specification limit (ppm)
1	Atenolol	*N*-Nitrosoatenolol	NNAT	4	1,500	200	7.500
2	Bisoprolol fumarate	*N*-Nitrosobisoprolol	NNBI	4	1,500	20	75.000
3	Carvedilol	*N*-Nitrosocarvedilol	NNCA	5	1,500	80	18.750
4	Labetalol HCl	*N*-Nitrosolabetalol	NNLA	4	1,500	2,400	0.625
5	Metoprolol fumarate	*N*-Nitrosometoprolol	NNME	4	1,500	400	3.750
6	Propranolol HCl	*N*-Nitrosopropranolol	NNPR	4	1,500	640	2.344

*Reference: RxList information on maximum daily dose

Table 2. Concentration of NDSRIs for LOD, LOQ, and linearity range calculated as per specification limit.

Concentration of NDSRIs								
					Absolute concentrations (ng/mL)			
Sr. no.	NDSRI	Drug substance	Sample conc. (mg/mL)	Specification limit (ppm)	LOD	LOQ	100% level	Linearity (10% to 350%)
1	NNAT	Atenolol	2	7.500	0.500	1.500	15.000	1.500–52.500
2	NNBI	Bisoprolol fumarate	2	75.000	5.000	15.000	150.000	15.000–525.000
3	NNCA	Carvedilol	2	18.750	1.250	3.750	37.500	3.750–131.250
4	NNLA	Labetalol HCl	10	0.625	0.210	0.625	6.250	0.625–21.874
5	NNME	Metoprolol fumarate	10	3.750	1.250	3.750	37.500	3.750–131.250
6	NNPR	Propranolol HCl	10	2.344	0.782	2.344	23.440	2.340–82.040

Table 3. Details of drug substance sample preparation.

Drug substance sample preparation						
Sr. no.	Drug substances	Related NDSRI	Required sample conc. (mg/mL) *For non-spiked / spiked sample preparation*	Volume (mL) made up to mark with *For non-spiked samples*	Volume (mL) made up to mark with *For spiked samples (recovery experiment)*	
1	Atenolol	NNAT	2	Diluent solution	10% Level solution	For Low-level spiking
2	Bisoprolol fumarate	NNBI	2			
3	Carvedilol	NNCA	2		100% Level solution	For Mid-level spiking
4	Labetalol HCl	NNLA	10			
5	Metoprolol fumarate	NNME	10		350% Level solution	For High-level spiking
6	Propranolol HCl	NNPR	10			

Chromatographic conditions

Table 4. HPLC conditions.

Parameter	Value			
HPLC column	Hypersil GOLD C18 Selectivity HPLC Column, 3 mm × 100 mm, 3 µm (P/N 25003-103030)			
Column temp.	40 °C			
Flow rate	0.400 mL/min			
Mobile phase A	0.1% formic acid in water			
Mobile phase B	0.1% formic acid in methanol			
Gradient	Time (min)	% A	% B	Curve
	0	80	20	5
	6	80	20	5
	7	60	40	5
	8	43	57	5
	9	43	57	5
	11	30	70	5
	12	20	80	5
	15	20	80	5
	15.2	80	20	5
	20	80	20	5
Sampler temp.	10 °C			
Needle wash	80:20, methanol:water			
Static mixer	150 µL			
UV wavelength	224 nm (for all 6 drug substances)			

Table 5. Injection volume for analysis of the NDSRIs.

NDSRI	NNAT	NNBI	NNCA	NNLA	NNME	NNPR
Inj. volume (µL)	4	2	3	3	2	2

Mass spectrometer settings

Table 6. Ion source settings.

Parameter	Value
Ion source type	H-ESI
Positive ion spray voltage	3,500 V
Sheath gas flow rate	42 arbitrary units
Aux gas flow rate	7 arbitrary units
Sweep gas flow rate	0 arbitrary unit
Ion transfer tube temp.	275 °C
Aux gas heater temp.	300 °C

Table 7. Divert valve settings.

Divert valve setup				
NNAT	Time (min)	0	7.8	10.5
	*Position	1-6	1-2	1-6
NNBI	Time (min)	0	10.5	14
	Position	1-6	1-2	1-6
NNCA	Time (min)	0	11	15
	Position	1-6	1-2	1-6
NNLA	Time (min)	0	10.8	15
	Position	1-6	1-2	1-6
NNME	Time (min)	0	9.5	13
	Position	1-6	1-2	1-6
NNPR	Time (min)	0	11	15.5
	Position	1-6	1-2	1-6
Note:	While creating an instrument method for analysis of an NDSRI, its corresponding divert valve setup can be referred.			
***Position**	1-2		Into MS	
	1-6		Diverted into UV or Waste	

MS properties

Table 8. Common parameters for all the NDSRIs in MS properties.

Parameter	Value
Expected LC peak width	12 s
Q1 resolution	3
Q1 offset	Off
Internal calibration	Off
CE mode	Fixed
CE type	Normalized
Orbitrap resolution	120,000
Scan range mode	Auto
Maximum injection time	Auto
Data type	Centroid

MS scan parameters

A product ion scan was performed to analyze NDSRIs, keeping the best optimized scan parameters for data acquisition as compiled in Table 9. This table can be referenced to create an instrument method for data acquisition of any of the NDSRIs to be determined in related drug substance.

Data processing

In the extracted ion chromatograms (XIC), the data was processed with a mass precision value in five decimal places for all six NDSRIs. A mass tolerance of 5 ppm was applied for NNCA and NNLA, while that of 10 ppm was applied for other NDSRIs to perform the quantitation. The observed retention time for NDSRIs and *m/z* values to be extracted for data processing are listed in Table 10.

Table 9. Product ion scan for six NDSRIs.

Sr. no.	Comp.	Mol. formula	*m/z*	Start time (min)	End time (min)	Collision energy (V)	RF (V)	Polarity
1	NNAT	$C_{14}H_{21}N_3O_4$	296.1605	8	10.5	10	60	Positive
2	NNBI	$C_{18}H_{30}N_2O_5$	355.2227	10.7	14	15	60	Positive
3	NNCA	$C_{24}H_{25}N_3O_5$	436.1869	11.2	15	10	70	Positive
4	NNLA	$C_{19}H_{23}N_3O_4$	358.1761	11	15	20	60	Positive
5	NNME	$C_{15}H_{24}N_2O_4$	297.1809	10	13	5	50	Positive
6	NNPR	$C_{16}H_{20}N_2O_3$	289.1547	11.2	15.5	15	60	Positive

Table 10. MS extraction parameters for NDSRIs.

NDSRI	Ret. time (min)	*m/z* to be extracted	Confirming ion (optional)
NNAT	9.68	222.13620	265.15467
NNBI	12.71	145.09715	72.08078
NNCA	12.9	180.10170, 253.11790	312.13427
NNLA	12.31	166.04987, 310.16780	180.06552
NNME	11.79	267.18280	223.15668
NNPR	13.4	145.09710	72.08078

Results and discussion

Method development and experimental challenges

For a reliable and long-term reproducible method, there are few checkpoints that need to be properly addressed during method development. With the NDSRIs under study, we faced a few challenges which were handled with the selection of an appropriate precursor ion through high-resolution scanning, RT confirmation with a high concentration of NDSRI neat solution injected onto the system, selection of product ions after confirmation through Mass Frontier 8.0 SR2 software (which helps with molecular ion fragmentation and knowing the exact *m/z* value for product ion masses), and ion ratio confirmation.

One example of this approach is shown in Figure 2 for NNBI. Figure 2A shows an NNBI chromatogram for retention time confirmation after injecting a higher concentration in full scan mode, keeping 355.22275 *m/z* for the extracted ion chromatogram. Figure 2B shows tMS data where there is a clear interference observed within 6 ppm mass tolerance. Figure 2C shows mass spectra from a tMS scan that gives the *m/z* of an interference at the precursor ion, and, therefore, the tMS2 scan was a better choice for data acquisition. Figure 3 shows an example of NNBI for theoretical fragmentation through Mass Frontier software to confirm the product ions with an accuracy up to the fifth decimal place. Similarly, the product ions for other NDSRIs were also confirmed.

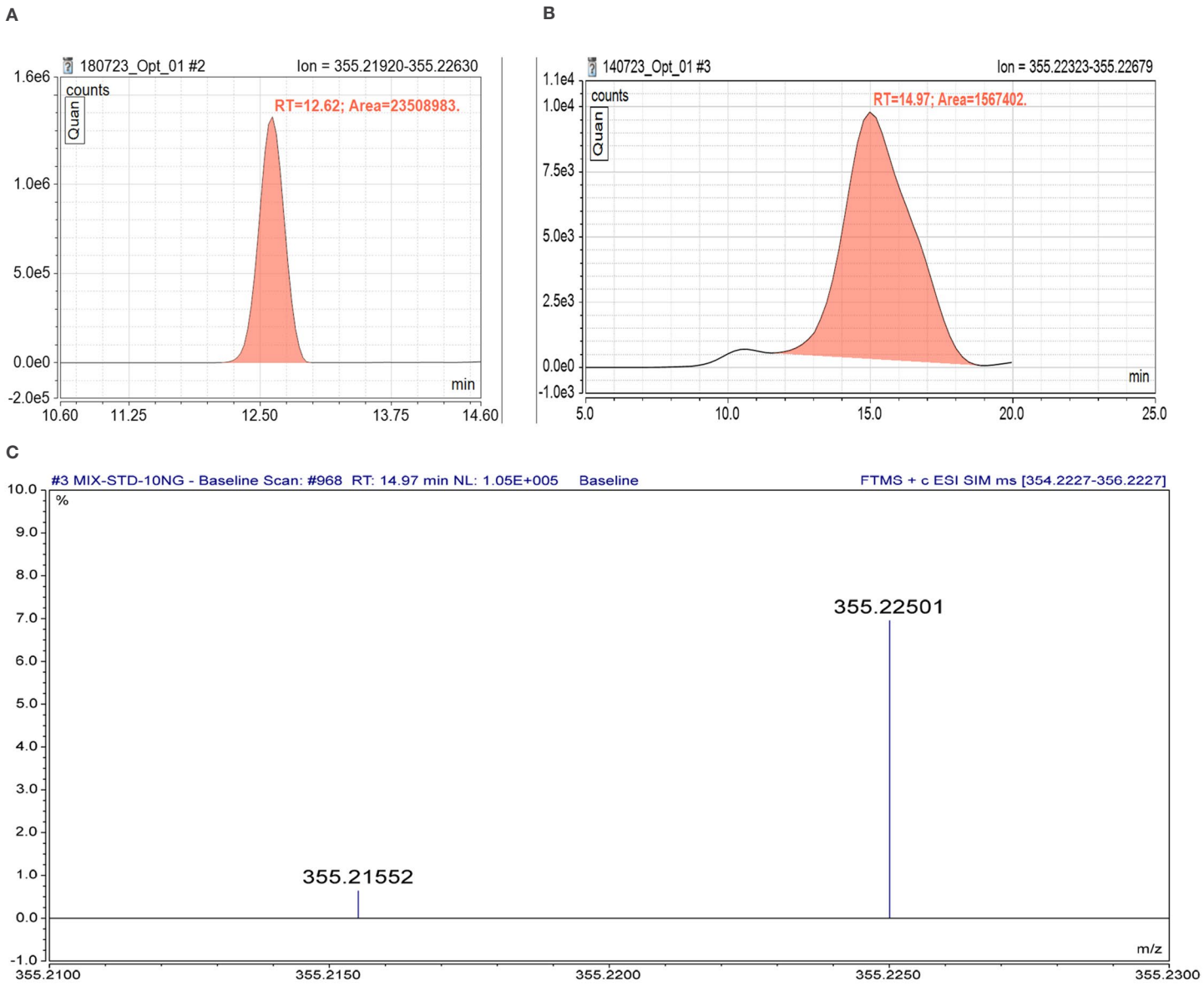


Figure 2. Precursor ion evaluation and product ion selection for NNBI. (A) A retention time of 12.62 minutes was evident from the peak extracted at the protonated precursor ion 355.22275 *m/z* from full scan MS data acquired at higher concentration. (B) Extracted ion chromatogram of 355.22501 *m/z* that interferes within 6 ppm mass tolerance. (C) Mass spectra showing very close interference at *m/z* 355.22501.

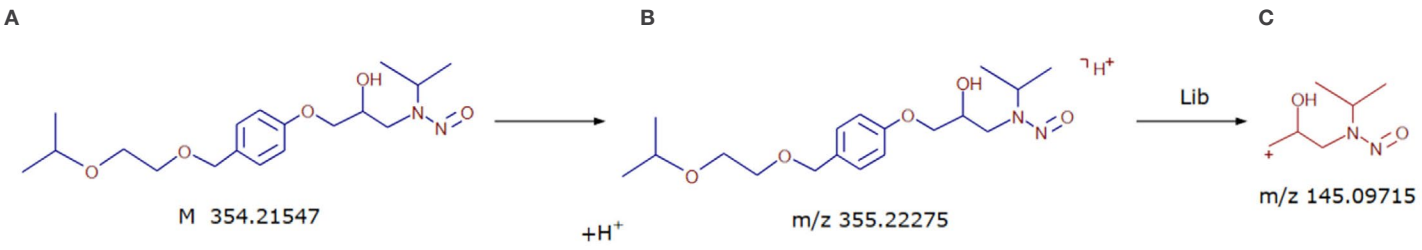


Figure 3. Mass Frontier software representation for accurate mass NNBI (A), precursor ion (B), product ion (C).

Suitable chromatography

The goal of developing a faster and more efficient method of analysis to determine any of the six NDSRIs in its respective drug substance was achieved after a series of optimizations with respect to chromatography and sample preparation. Highly resolved chromatography between a drug substance and a related NDSRI within a short runtime of 20 minutes was achieved using the Hypersil GOLD C18 Selectivity, 100 × 3 mm, 3 µm, chromatography column. Mobile phase selection was also based upon optimum ionization and better than minimum required signal-to-noise ratio (S/N) with appropriate chromatographic peak shape of NDSRIs.

Appropriate ion selection

Based upon the observations, most of the NDSRIs under study were found to have a high tendency to form multiple adducts (e.g., $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$). This adduct ion formation may be due to multiple reasons, but one common challenge in using a non-protonated form of molecular ions is to achieve consistency in peak area response obtained over a period. Therefore, to select the most appropriate ions, a full scan of the precursor ion was acquired to confirm the retention time of the molecule and was compared to what is obtained in the product ion scan. Afterwards, multiple product ions were selected to confirm response, reproducibility, and linearity. The best product ions were then confirmed with the ones suggested by Mass Frontier 8.0 SR2 software.

Ion ratio confirmation

To further verify data reliability, the ion ratio confirmation was calculated between the qualifier ion and the quantifier ion/ions. Product ion selection for each NDSRI was therefore confirmed with the successful data obtained from the ion ratio confirmation exercise as per the European Commission Decision (2002/657/EC)[6]. Table 11 shows the ion ratio confirmation, and its results obtained with all six NDSRIs.

Experimental design

As a verification of accuracy and robustness of the method, multiple experiments were performed. To keep uniformity during method evaluation, each NDSRI was analyzed in its corresponding drug substance with similar acquisition sequence / injection order. The experiments and the injection order are described below:

Reproducibility, LOD, LOQ, linearity, and recovery:

1. Diluent blank injection at the beginning of the sequence
2. Six replicate injections of standard solution at specification level followed by one injection of diluent blank
3. Three replicate injections of LOD
4. Six replicate injections of LOQ
5. Eight levels of linearity standards, each in triplicate in increasing order from 10% to 350% level followed by one injection of diluent blank and one injection of bracketing standard (100% specification level)
6. Three replicate injections of non-spiked drug substance sample
7. Three replicate injections each of drug substance sample spiked at three different levels (10%, 100%, and 350% of the specification level) followed by one injection of the bracketing standard and diluent blank.

Table 11. Ion ratio confirmation results.

NDSRI	API	Target ion ratio criteria	Target ratio - qualifier to quantifier*	Ion ratio obtained in samples**	Status
NNAT	Atenolol	± 25%	45.22	45.43	Confirmed
NNBI	Bisoprolol fumarate	± 50%	3.26	3.26	Confirmed
NNCA	Carvedilol	± 20%	69.82	71.88	Confirmed
NNLA	Labetalol HCl	± 50%	6.33	6.50	Confirmed
NNME	Metoprolol fumarate	± 20%	156.43	155.3	Confirmed
NNPR	Propranolol HCl	± 20%	95.04	95.49	Confirmed

*Mean value of ion ratio obtained from all linearity standards

**Mean value of ion ratio obtained from API samples

Experimental results

Excellent results were obtained for each NDSRI with respect to all activities performed with neat standards as well as non-spiked and spiked samples of the respective drug substance in this method evaluation. Data processing and calculations were performed utilizing the comprehensive and versatile features of Chromeleon CDS.

Standard solution reproducibility (specification level)

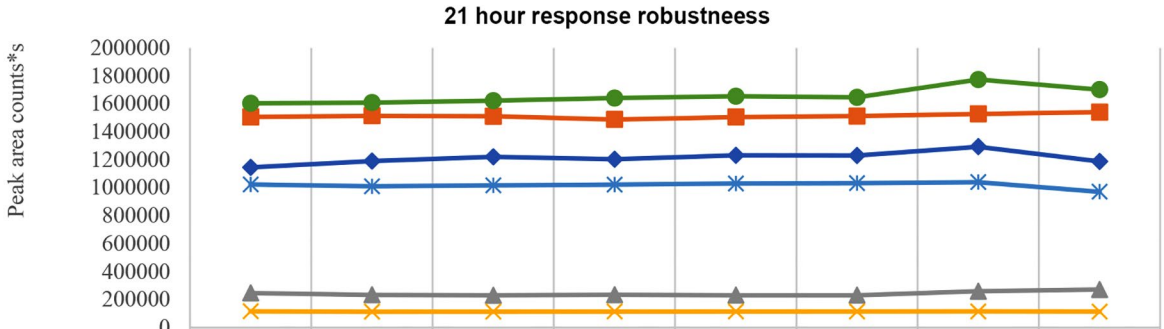
Data acquired for the reproducibility experiment with six replicate injections of a specification level standard solution were found to be well within the general acceptance criteria and are tabulated for all six NDSRIs in Table 12.

Robustness results (specification level standard solution with bracketing standards)

Robustness was calculated in terms of reproducibility using the specification level standard solution acquired in start and including the interspersed as well as the last injection of the bracketing standard of the sequence. The robustness plot and the calculations are shown in Figure 4.

Table 12. Reproducibility at 100% specification level concentration for all NDSRIs.

Standard solution reproducibility (specification level)						
Sample name	NNAT area (cps)	NNBI area (cps)	NNCA area (cps)	NNLA area (cps)	NNME area (cps)	NNPR area (cps)
STD-100% Level - 1	1,146,240	1,146,240	1,146,240	117,083	1,0234,26	1,604,777
STD-100% Level - 2	1,191,123	1,191,123	1,191,123	114,925	1,011,027	1,609,188
STD-100% Level - 3	1,221,317	1,221,317	1,221,317	114,827	1,017,079	1,623,296
STD-100% Level - 4	1,204,233	1,204,233	1,204,233	115,483	1,022,414	1,642,198
STD-100% Level - 5	1,232,198	1,232,198	1,232,198	116,020	1,030,095	1,655,280
STD-100% Level - 6	1,230,183	1,230,183	1,230,183	115,412	1,033,394	1,646,951
Mean	**1,204,216**	**1,204,216**	**1,204,216**	**115,625**	**1,022,906**	**1,630,282**
SD	**32,503.4**	**32,503.4**	**32,503.4**	**833.6**	**8212.9**	**20,923.7**
% RSD	**2.7**	**2.7**	**2.7**	**0.7**	**0.8**	**1.3**



	STD-100% Level - 1 (Inj#3)	STD-100% Level - 2 (Inj#4)	STD-100% Level - 3 (Inj#5)	STD-100% Level - 4 (Inj#6)	STD-100% Level - 5 (Inj#7)	STD-100% Level - 6 (Inj#8)	Bracketing STD-100% Level - 1 (Inj#47)	Bracketing STD-100% Level - 2 (Inj#61)
NNAT; % RSD = 3.5	1146240	1191123	1221317	1204233	1232198	1230183	1293756	1187801
NNBI; % RSD = 1.0	1506825	1515215	1511795	1489073	1505885	1513582	1528255	1541075
NNCA; % RSD = 6.4	248273	235221	231904	235844	232858	231926	260920	272914
NNLA; % RSD = 0.7	117083	114925	114827	115483	116020	115412	116428	114784
NNME; % RSD = 2.1	1023426	1011027	1017079	1022414	1030095	1033394	1040881	971583
NNPR; % RSD = 3.4	1604777	1609188	1623296	1642198	1655280	1646951	1774102	1701916

Figure 4. Representation of peak area response robustness. Each NDSRI has shown good reproducibility including bracketing standard injections in individual sequences.

LOD data and LOQ reproducibility

LOD was run in triplicate, LOQ was run in six replicates, and the S/N was observed. Since the data was acquired with high resolution and processed with an appropriate mass tolerance, the chemical noise is minimized and the S/N is drastically improved, even at very low concentration levels.

S/N was calculated with the formula: 2H/h

Where:

H = height of the main peak

h = height of the noise region

The data tabulated for all six NDSRIs in a consolidated form can be viewed in Table 13 which includes LOD injections and S/N. Similarly, Table 14 shows LOQ reproducibility and S/N results.

Linearity

Linearity was drawn from the average peak area response of triplicate injections of each of the eight calibration standards. Calibration curves are shown in Figure 5. Linearity data is shown in Table 15 that additionally mentions reproducibility at each calibration level.

Recovery

Extraction efficiency and accuracy obtained in the spiked samples were very good and perfectly comparable to non-spiked samples and neat standards to calculate percentage recovery. The data has been tabulated for all six NDSRIs in Table 16.

Table 13. Consolidated data representation of peak area response of LOD from triplicate injections along with the S/N values for all NDSRIs.

Limit of detection (LOD)												
	NNAT		NNBI		NNCA		NNLA		NNME		NNPR	
Inj #	Area (cps)	S/N	Area (cps)	S/N	Area (cps)	S/N	Area (cps)	S/N	Area (cps)	S/N	Area (cps)	S/N
1	39,360	784	54,253	20,142	6,599	485	3,080	432	36,163	111	53,501	102
2	39,192	781	52,620	19,183	7,188	540	3,679	483	35,415	107	56,496	106
3	38,074	762	53,559	19,773	5,026	372	3,771	526	36,045	111	57,227	110

Table 14. LOQ results representation on basis of peak area response and S/N for all NDSRIs.

Limit of quantitation (LOQ)									
	NNAT			NNBI			NNCA		
Inj #	RT (min)	Area (cps)	S/N	RT (min)	Area (cps)	S/N	RT (min)	Area (cps)	S/N
1	9.68	119,550	2,398	12.69	159,330	5,6624	12.95	23,774	1,761
2	9.68	118,967	2,388	12.71	159,837	5,8625	12.93	23,320	1,718
3	9.68	120,077	2,396	12.71	160,444	5,8993	12.93	22,671	1,686
4	9.68	120,825	2,436	12.71	161,400	6,0239	12.92	23,249	1,710
5	9.68	123,270	2,473	12.71	159,751	5,8701	12.92	23,453	1,741
6	9.68	116,756	2,307	12.71	160,205	5,9399	12.93	22,317	1,664
Mean	**9.68**	**119,908**		**12.71**	**160,161**		**12.93**	**23,131**	
SD	**0.00**	**2,150.4**		**0.01**	**718.4**		**0.01**	**536.8**	
% RSD	**0.0**	**1.8**		**0.1**	**0.4**		**0.1**	**2.3**	
	NNLA			**NNME**			**NNPR**		
Inj #	**RT (min)**	**Area (cps)**	**S/N**	**RT (min)**	**Area (cps)**	**S/N**	**RT (min)**	**Area (cps)**	**S/N**
1	12.31	12,352	1,571	11.79	106,115	324	13.41	168,535	321
2	12.31	11,601	1,521	11.79	104,252	321	13.41	166,917	321
3	12.31	11,862	1,533	11.79	107,482	337	13.41	167,848	316
4	12.31	12,061	1,626	11.79	108,186	336	13.41	168,812	320
5	12.31	12,305	1,598	11.81	107,293	328	13.40	171,107	321
6	12.31	11,404	1,492	11.79	107,320	336	13.41	169,816	324
Mean	**12.31**	**11,931**		**11.79**	**106,775**		**13.41**	**168,839**	
SD	**0.00**	**380.9**		**0.01**	**1,404.1**		**0.00**	**1,474.3**	
% RSD	**0.0**	**3.2**		**0.1**	**1.3**		**0.0**	**0.9**	

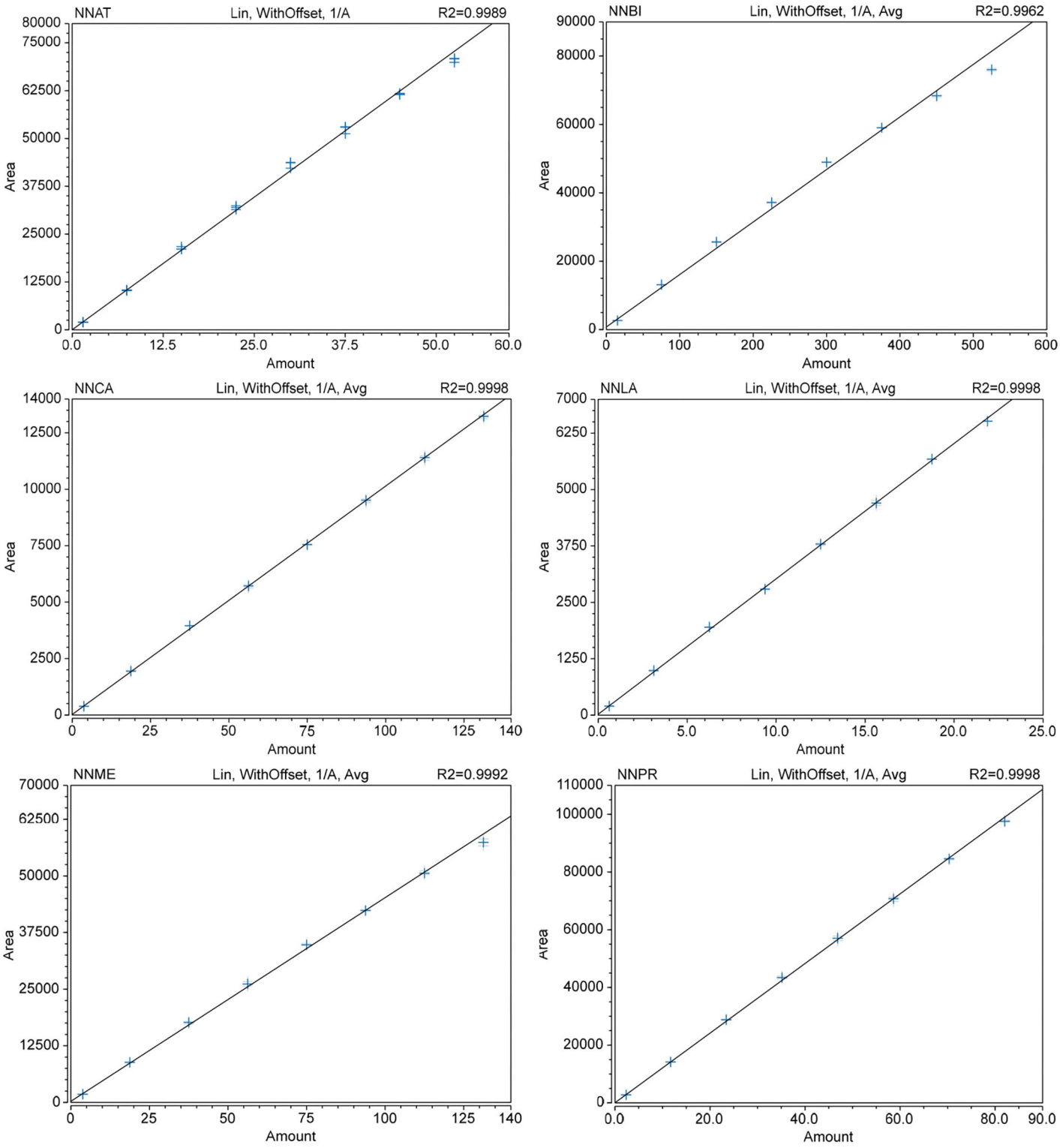


Figure 5. Calibration curve plots of each NDSRI from the linearity experiment along with values of coefficient of determination (R^2).

Table 15. Data of eight calibration levels acquired in triplicate in the linearity experiment. Representation of mean peak area and reproducibility at each calibration level.

Mean response and reproducibility of eight calibration levels acquired in triplicate in linearity experiment									
Calibration level	Name	Average area (cps)	%RSD of triplicates	Name	Average area (cps)	%RSD of triplicates	Name	Average area (cps)	%RSD of triplicates
10% Level	NNAT	119,913	2.1	NNBI	159,597	0.7	NNCA	22996	3.3
50% Level	NNAT	620,480	1.0	NNBI	787,766	0.6	NNCA	116366	1.9
100% Level	NNAT	1,277,839	1.7	NNBI	1,538,164	0.6	NNCA	237354	0.2
150% Level	NNAT	1,917,056	1.6	NNBI	2,230,133	0.5	NNCA	342833	1.3
200% Level	NNAT	2,592,691	2.0	NNBI	2,9361,28	0.4	NNCA	452910	0.3
250% Level	NNAT	3,144,130	1.9	NNBI	3,542,386	0.2	NNCA	570643	1.0
300% Level	NNAT	3,695,346	0.3	NNBI	4,101,438	0.5	NNCA	684047	0.9
350% Level	NNAT	4,231,721	0.8	NNBI	4,561,837	0.6	NNCA	793636	0.6
Calibration level	Name	Average area (cps)	%RSD of triplicates	Name	Average area (cps)	%RSD of triplicates	Name	Average area (cps)	%RSD of triplicates
10% Level	NNLA	11,923	3.9	NNME	107,248	1.4	NNPR	167,040	1.4
50% Level	NNLA	59,053	2.0	NNME	530,183	0.9	NNPR	850,487	0.3
100% Level	NNLA	116,968	0.4	NNME	1,058,753	1.7	NNPR	1728,462	0.7
150% Level	NNLA	167,459	0.4	NNME	1,569,353	1.9	NNPR	2,606,119	1.1
200% Level	NNLA	227,366	0.7	NNME	2,088,211	0.7	NNPR	3,422,604	1.4
250% Level	NNLA	282,061	1.3	NNME	2,542,181	0.3	NNPR	4,240,712	1.1
300% Level	NNLA	340,493	0.2	NNME	3,037,394	0.9	NNPR	5,074,377	0.3
350% Level	NNLA	390,988	0.1	NNME	3,445,049	1.3	NNPR	5,854,499	0.3

Table 16. Recovery results at low, mid, and high concentrations for six NDSRIs.

Recovery experiment results calculated with mean peak area response of each non-spiked and spiked sample acquired in triplicate							
Concentration level	Level with respect to specification limit	Recovery percentage					
		NNAT	NNBI	NNCA	NNLA	NNME	NNPR
Low	**10%**	91.7	99.6	106.7	77.6	93.2	94.6
Mid	**100%**	97.6	101.9	96.1	77.8	94.8	91.0
High	**350%**	88.0	98.2	85.4	77.1	92.8	85.2

Calculation to determine impurity content as per the FDA[7]

Drug substance: $$\text{Nitrosamine impurity (ppm)} = \frac{A_{spl}}{A_s} \times C_s \times \frac{1\text{ mg}}{1 \times 10^6\text{ ng}} \times \frac{V}{W} \times 10^6$$

Where:

Nitrosamine impurity refers to each impurity individually

A_{spl} = Area of the nitrosamine impurity peak in the sample solution

A_s = Average area (n = 6) of the nitrosamine impurity peak from the first six consecutive injections of the standard solution

C_s = Concentration of the nitrosamine impurity in the standard solution (3.0 ng/mL)

W = Weight of drug substances (mg)

V = Volume of the diluent in the sample solution (mL)

Chromatography results

Excellent chromatography results were obtained for LOD, LOQ, specification level standard, non-spiked samples, and spiked samples. Representative chromatograms are presented in Figure 6 for all APIs and Figure 7 for diluent blank, LOD, LOQ, and 100% specification level standards. The chromatography obtained in this method is achieved by using a Vanquish Flex Binary UHPLC system with a Hypersil GOLD C18 Selectivity, 100 × 3 mm, 3 µm, HPLC column and the efficient gradient program of 20 minutes to perform inevitable chromatographic separation between impurity and API and confidently determine the content of an NDSRI in related drug substance.

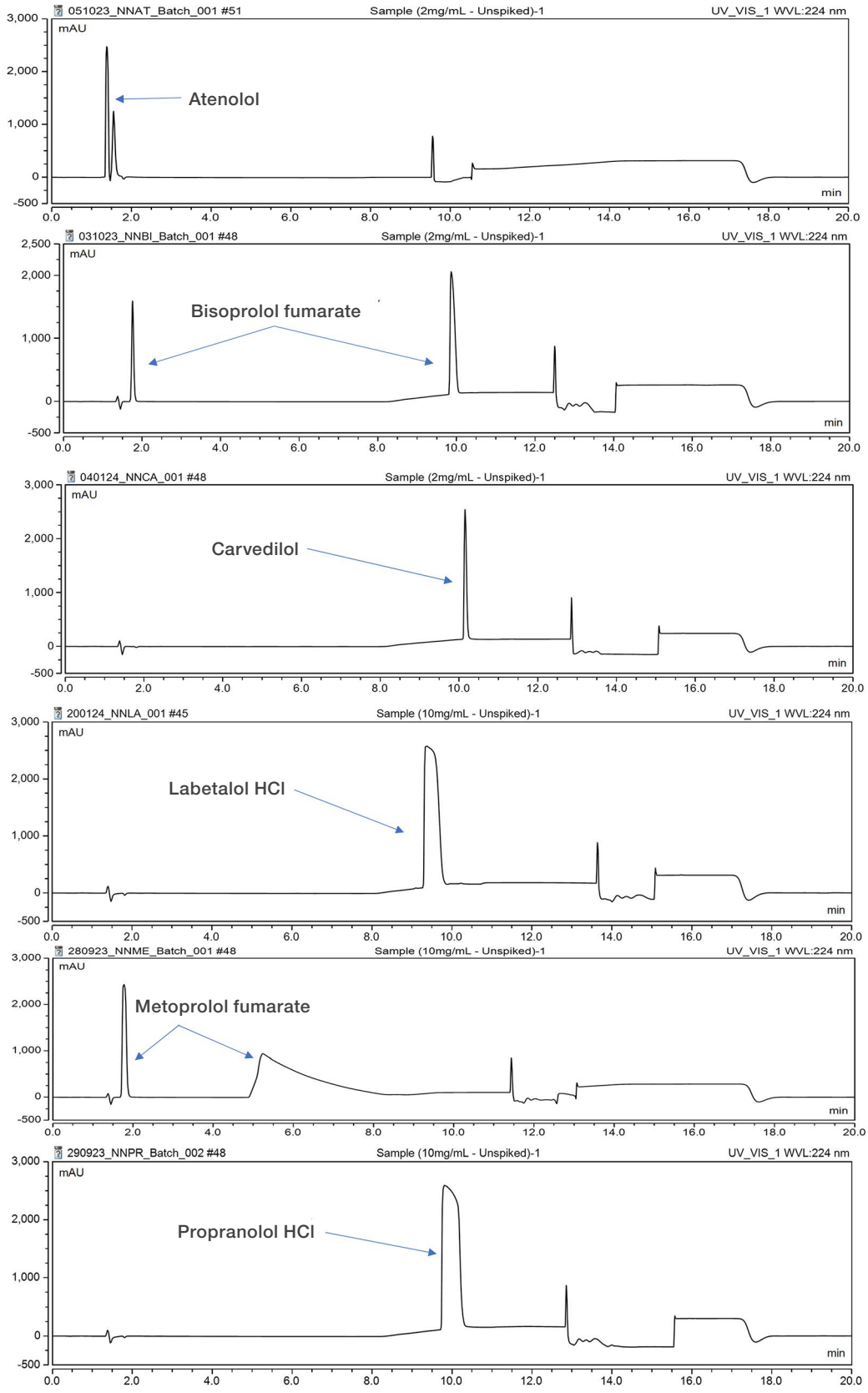


Figure 6. Chromatography in UV at 224 nm for drug substances.

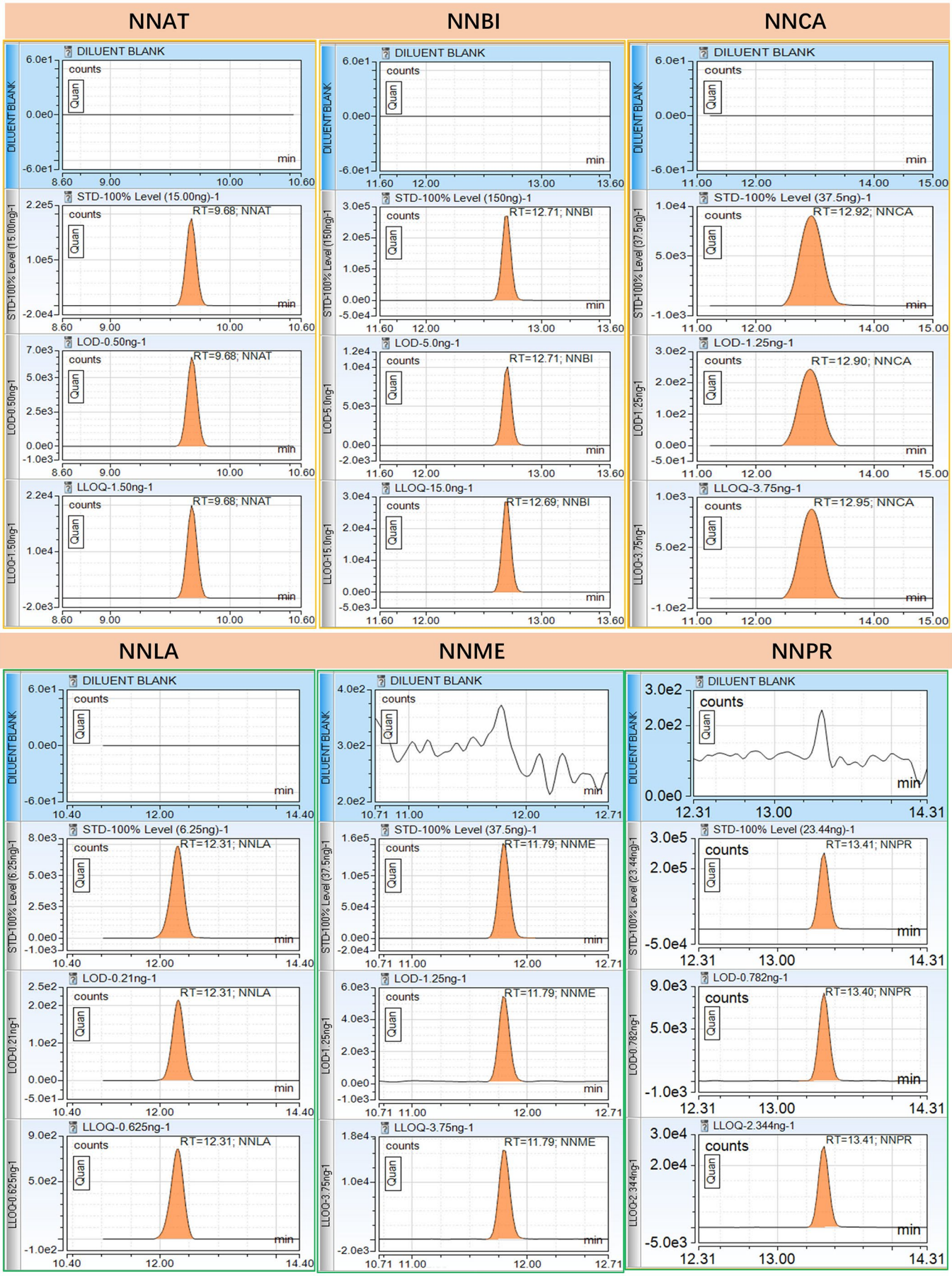


Figure 7. Chromatography of diluent blank, 100% specification level standard, LOD, LOQ.

Results summary

All experiments were evaluated to meet quality standards of analysis to ensure that the method developed in this work is accurate, reliable, and reproducible in terms of accuracy, precision, linearity, recovery, and ion ratio confirmation. Table 17 shows consolidated results to showcase the performance with the experimental values and ranges observed from standards and samples.

Table 17. Consolidated experimental results.

Consolidated results						
Activity	Reproducibility	Response robustness	LOD and LOQ	Linearity	Recovery at low, mid and high levels	Ion ratio confirmation (optional)
NDSRI	Specification & LOQ level	Specification level	Signal-to-noise	10%-350% levels		
NNAT	Maximum *RSD observed = 3.2%	Maximum *RSD observed = 6.4% with bracketing standards	Minimum S/N observed in LOD = 102	Minimum R^2 observed = 0.9962	Recovery observed = 77.1% to 106.7%	Ion ratio confirmation in standards and samples at concentration ≥ LOQ was found to be 'Ok'
NNBI						
NNCA						
NNLA						
NNME						
NNPR						

*Relative standard deviation

Conclusion

A versatile LC-HRMS-based methodology for the quantitative analysis of NDSRIs in six corresponding beta-blocker drug substances has been successfully developed with excellent data of reproducibility, linearity, long run robustness, recovery, and ion ratio confirmation. The method showed capabilities to meet expectations for all six NDSRIs in terms of sensitivity and S/N for even lower concentration levels calculated based upon the CPCA approach. Important highlights of the method are mentioned below:

- The R^2 values obtained from calibration curve linearity for all NDSRIs were >0.99.
- The LOQ for all NDSRIs corresponds to a concentration that is 10% of the specification limit to meet regulatory requirements and an excellent value of signal-to-noise ratio has also been obtained. The maximum relative standard deviation for six replicate injections of LOQ among all six NDSRIs was found to be 3.2%.
- The LOD corresponds to a concentration that is 1/3 the LOQ for all six NDSRIs. The minimum signal-to-noise ratio obtained among all six NDSRIs at LOD level was excellent with a value of 102.
- Recovery results were found to be well within the 70%–130% acceptance criteria.
- Additional activity of ion ratio confirmation was also performed to validate correct selection of product ions for all six NDSRIs. The ion ratio confirmation experiment was found to be successful with confirmation for all standards and samples.
- A cumulative %RSD (including system suitability standard and bracketing standards) or long run robustness experiment was successful with maximum %RSD of 6.4% for all six NDSRIs when the entire sequence was analyzed for 21 hours continuously, which reflects the robustness of the system and method suitability.
- The Orbitrap Exploris 120 mass spectrometer with the Vanquish Flex Binary system proves its capability while achieving the desired data output for the six NDSRIs. The sensitivity, resolution, and robustness of the instrument and method meet expectations for the determination of the NDSRIs that are related to six beta blocker-drug substances with one versatile method approach. Chromeleon CDS and Mass Frontier software together form a powerful workflow for NDSRI quantitation and accurate ion selection.
- This method has an excellent versatile benefit of applying for quantitation of any of the NDSRIs in six related beta-blocker drug substances, namely atenolol, bisoprolol fumarate, carvedilol, labetalol HCl, metoprolol fumarate and propranolol HCl within a runtime of 20 minutes.

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