DETERMINATION OF N-NITROSO SALBUTAMOL IN SALBUTAMOL PRODUCTS BY LC-MS/MS

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Disclaimer: The testing method below provides option and guidance for the users to determine N-Nitroso Salbutamol in Salbutamol drug products. The method should be validated by users to ensure it is fit for its intended use.

1 Scope

This document outlines the test method for the determination of N-Nitroso Salbutamol ($C_{13}H_{20}N_2O_4$) in Salbutamol drug products by Liquid Chromatography Hybrid Tandem Mass Spectrometry (LC-MS/MS).

2 Determination of N-Nitroso Salbutamol by LC-MS/MS

2.1 Reagents and Chemicals

N-Nitroso Salbutamol (Purity ≥ 97%)
Methanol (MeOH), HPLC grade
Formic acid, MS grade
Deionized water (DI water)

2.2 Instruments and Apparatus

Liquid Chromatography Tandem Mass Spectrometry (QTRAP 7500 MS/MS coupled with ExionLC)
Centrifuge
Ultrasonic bath
Volumetric flask (Class A, 10 and 50 mL)
Membrane syringe filter (Nylon 0.2 µm)
Micropipette
2 mL vials
Conical bottom centrifuge tube, Polypropylene (PP)

2.3 LC-MS/MS Method

HPLC

Column:	Hypersil GOLD analytical column (150×2.1 mm, 3 µm) or equivalent
Column oven temperature:	40 °C
Injection volume:	5 µL
Mobile phase A:	0.1% formic acid in DI water
Mobile phase B:	0.1% formic acid in Methanol
Flow rate:	0.3 mL/min

Gradient:	Time (min)	Mobile phase A (%)	Mobile phase B (%)
	0	80	20
	5.0	80	20
	8.0	5	95
	11.0	5	95
	11.5	80	20
	15	80	20

[Note: The flow rate or run time may be varied to obtain optimum separation.]

MS/MS

MS:	QTRAP 7500
Ionization mode:	ESI (Electrospray ionization), Negative
MS parameter:	CUR: 40 psi; TEP: 500 °C; GS 1: 50 psi GS 2: 70 psi; IS: 2500

Valve switches*:	Time (min)		Position	Remark
	0.0-7.5		A	To waste
	7.5-9.0		B	To MS
	9.0-15.0		A	To waste

MRM:	ID	Q1	Q3	EP	CE
	N-Salbutamol 1	267.1	151.1	-10	-30
	N-Salbutamol 2	267.1	219.2	-10	-22
	N-Salbutamol 3	267.1	162.9	-10	-28

[*Note: Valve switches window may be adjusted depending on the different system to avoid excessive contamination of MS detector from API and excipients (subject to the RTs of the target analytes)]

2.4 Standard, Sample, Sample Blank and Spiked Sample Preparation

2.4.1 Standard Preparation

1. *Stock Standard Solution* (100 µg/mL): Prepare from commercially available standard and dilute with methanol.
2. *Intermediate Standard Solution I* (10 µg/mL): Accurately transfer 1 mL of *Stock Standard Solution* to a 10 mL volumetric flask and top up to volume using DI water.
3. *Intermediate Standard Solution II* (100 ng/mL): Accurately transfer 100 µL of *Stock Standard Solution I* to a 10 mL volumetric flask and top up to volume using DI water.
4. *Working Standard Solution*s (prepared in volumetric flask individually):

Working Standard Solution	Standards Conc.	Vol of *Int Standard Solution II* (100 ng/mL)	Top up to Volume using DI water
1	0.1 ng/mL	50 µL	50 mL
2	0.2 ng/mL	20 µL	10 mL
3	1 ng/mL	100 µL	10 mL
4	5 ng/mL	2500 µL	50 mL
5	10 ng/mL	1000 µL	10 mL
6	20 ng/mL	2000 µL	10 mL

2.4.2 Sample Preparation

1. Weigh 10 units of sample together and calculate the average weight per unit.
2. Accurately weigh an amount of powdered sample, equivalent to 10 mg of drug substance into a 15 mL PP conical bottom centrifuge tube.
3. Add 10 mL DI water, vortex to mix well and sonicate for 10 min.
4. Centrifuge at 3500 rpm for 5 min.
5. Filter the supernatant into a HPLC vial through a 0.2 µm Nylon membrane filter.

[Notes]

1. Scale down the sample amount if necessary.
2. In the situation when the amount of powder equivalent to 10 mg drug substance is too much for effective sample extraction, reduce the powder amount to not more than 1 g. In this case, the LOD of the method will be affected and needed to be recalculated accordingly.

2.4.3 Sample Blank Preparation

Sample Blank Solution: Prepare the Sample Blank as described for the Sample Preparation in Section 2.4.2 but without the sample addition.

2.4.4 Spiked Sample Preparation (Negative sample)

Spiked Sample Solution I (LOD spike, applicable for negative sample): Prepare as described for the Sample Preparation in Section 2.4.2, with addition of 10 mL of *Working Standard Solution 1* (0.1 ng/mL) instead of DI water to obtain *Spiked Sample Solution I* with 0.1 ng/mL spike of N-Nitroso Salbutamol (corresponding to 0.1 µg/g in sample with respect to Salbutamol drug substance).

Spiked Sample Solution II (5 ng/mL spike, applicable for recovery calculation): Prepare as described for the Sample Preparation in Section 2.4.2, with addition of 10 mL of *Working Standard Solution 4* (5 ng/mL) instead of DI water to Sample to obtain *Spiked Sample Solution II* with 5 ng/mL spike of N-Nitroso Salbutamol (corresponding to 0.5 µg/g in sample with respect to Salbutamol drug substance).

[Notes]
1. The sample may need to scale down or dilute further with DI water before spiking, so that the final concentration in *Spiked Sample Solution II* is within the linear plot.
2. For *Spiked Sample Solution II* preparation, the concentration of the *Standard Solution* added should be adjusted according to the dilution factor if further dilution was applied (e.g. 5 ng/mL × Dilution factor).

2.5 Test Procedure

1. Select method: N-Nitroso Salbutamol_*MRM*.
2. Inject solvent blank (DI water).
3. Inject *Working Standard Solution 1* (System sensitivity check), proceed if the S/N ratio of transition 267.1/162.9 is not less than 5
4. Inject *Working Standard Solution 2-6* (Duplicate).
5. Inject solvent blank.
6. Inject *Sample Blank*.
7. Inject *Sample Solutions* (Duplicate).
8. Inject *Spiked Sample Solution I.*
9. Inject *Spiked Sample Solution II* (Duplicate).
10. Inject solvent blank.
11. Inject *Working Standard Solution 2* (System performance check).
12. Flush LC-MS/MS system immediately after the analysis.

2.6 Interpretation of Results

1. For negative identification, the result is valid only if:
   i. No peaks corresponding to N-Nitroso Salbutamol was observed in the chromatogram obtained from the *Sample Solution.* Positive results are

obtained in *Spiked Sample Solution I*.

ii. The deviation of the peak area obtained from *Working Standard Solution 2* in Section 2.5 Step 4 and Step 11 (System performance check) should be not more than 20%.

iii. Report as 'Not Detected' and indicate the LOD of 0.1 µg/g (with respective to Salbutamol drug substance).

2. For positive identification, the result is valid only if:
   i. The peaks corresponding to N-Nitroso Salbutamol in the chromatogram of all the transitions from the *Sample Solution* have close retention time (±0.3 min) to the peaks from the *Standard Solutions* chromatogram.
   ii. The deviation of the peak area obtained from *Working Standard Solution 2* in Section 2.5 Step 4 and Step 11 (System performance check) should be not more than 20%.
   iii. The deviation of the peak area ratios of the three MRM transitions of N-Nitroso Salbutamol obtained from the *Standard Solutions* and *Sample Solution* should be not more than 20%.

3. The quantification is performed using the peak area of N-Nitroso Salbutamol: (267.1/151.0) through linearity plot from *Working Standard Solutions 2-6*. The quantification result is valid only if:
   i. The deviation of the peak area ratio of N-Nitroso Salbutamol: (267.1/151.0) obtained from duplicated sample solution are not more than 20%.
   ii. The linearity coefficient of the calibration plot is greater than 0.99.
   iii. The Recovery% obtained from *Spiked Sample Solution II* should be within the range of 70-125%.
   iv. Report as 'Less than LOQ' and indicate the LOQ of 0.2 µg/g (with respective to Salbutamol drug substance) if the peak area of N-Nitroso Salbutamol: (267.1/151.0) is above the peak area of *Standard Solution* of 0.1 ng/mL but less than the peak area of *Standard Solution* of 0.2 ng/mL.

2.7 Calculation

1. Calculation of N-Nitroso Salbutamol content in sample with respect to drug products (per unit)

   Content of N-Nitroso Salbutamol in drug product = $[(C_{Spl} \times V_{Spl} \times Dil) / W_{Spl}] \times W_{Unit}$

   Where:

   Content of N-Nitroso Salbutamol in drug product: ng/unit

   C_{Spl} : Concentration of N-Nitroso Salbutamol obtained from liner plot, ng/mL

   V_{Spl} : Volume of *Sample Solution*, mL

   W_{Spl} : Weight of sample used for sample preparation, g

   W_{Unit} : Average weight of each unit of sample, g (e.g. g/tab or g/cap etc.)

   Dil : Dilution factor

2. Calculation of N-Nitroso Salbutamol content in sample with respect to drug substance

Content of N-Nitroso Salbutamol with respect to drug substance = $[(C_{Spl} \times V_{Spl} \times Dil) / W_{Spl}] \times W_{Unit} / S_{Unit}$

Where:

Content of N-Nitroso Salbutamol with respect to drug substance: ng/g

C_{Spl} : Concentration of N-Nitroso Salbutamol obtained from liner plot, ng/mL

V_{Spl} : Volume of *Sample Solution*, mL

W_{Spl} : Weight of sample used for sample preparation, g

W_{Unit} : Average weight of each unit of sample, g (e.g. g/tab or g/cap etc.)

S_{Unit} : Strength of the drug product per unit, g (e.g. 0.1 for 100 mg/tab)

Dil : Dilution factor

3. Calculation of Recovery

Recovery% = $[(C_{Spiked\ sample} \times V_{Sample} \times Dil) - (Content_{Sample} \times W_{Spiked\ sample})] / [Conc_{spiked\ standard} \times Vol_{spiked\ standard}]$

Where:

$C_{Spiked\ sample}$: Concentration of N-Nitroso Salbutamol obtained from liner plot, ng/mL

V_{Sample} : Volume of *Sample Solution*, mL

$Content_{Sample}$: Content of N-Nitroso Salbutamol in sample obtained from liner plot, ng/g

$W_{Spiked\ sample}$: Weight of spiked sample used, g

$C_{Spiked\ standard}$: Concentration of N-Nitroso Salbutamol in the Spiked Standard Solution, ng/mL

$V_{Spiked\ standard}$: Volume of the Spiked Standard Solution used, mL

Dil : Dilution factor

4. Calculation of method LOD and LOQ with respect to drug substance

The instrument LOD and LOQ are 0.1 ng/mL and 0.2 ng/mL respectively. The corresponding method LOD and LOQ are 0.1 µg/g and 0.2 µg/g according to the sample preparation (sample amount used is equivalent to 10 mg of drug substance).

When the amount of sample used for sample preparation is not equivalent to 10 mg, the actual method LOD and LOQ should be calculated as below:

Method LOD (µg/g) = $(0.1 \times V_{Spl}) / A_{Drug\ substance}$

Method LOQ (µg/g) = $(0.2 \times V_{Spl}) / A_{Drug\ substance}$

Where:

0.1	:	Instrument LOD, ng/mL
0.2	:	Instrument LOQ, ng/mL
V_{Spl}	:	Volume of *Sample Solution*, mL
$A_{Drug\ substance}$	:	Amount of drug substance used for sample preparation, g (e.g. 0.01 g for drug substance in standard sample preparation in section 2.4.2)

Annex 1. MRM Chromatograms of N-Nitroso Salbutamol (1 ng/mL, Q-Trap 7500)