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Method of Test for 7-Nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-*a*]pyrazine (NTTP) in Sitagliptin Drug Substance

1. Scope

This method is applicable to the determination of 7-Nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-*a*]pyrazine (NTTP) in sitagliptin drug substances.

2. Method

After extraction, NTTP is determined by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: Hypersil GOLD C8, 3.0 μm, 4.6 mm i.d. × 10 cm, or an equivalent product.

2.1.2. Ultrasonicator.

2.1.3. Vortex mixer.

2.1.4. Centrifuge: centrifugal force ≥ 3000 ×g.

2.2. Chemicals

Methanol, HPLC grade;
Acetonitrile, HPLC grade;
Ammonium formate, reagent grade;
Deionized water, resistivity ≥ 18 MΩ · cm at (25°C);
7-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-*a*]pyrazine (NTTP), reference standard;
7-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-*a*]pyrazine-d_4 (NTTP-d_4) isotope-labeled internal standard.

2.3. Apparatus

2.3.1. Volumetric flask: 10 mL, amber flask.

2.3.2. Centrifuge tube: 15 mL, PP.

2.3.3. Membrane filter: 0.22 μm, PVDF.

2.4. Mobile phase

2.4.1. Solvent A:

Accurately weigh 0.63 g of ammonium formate and transfer into a 1 L

volumetric flask and dilute to volume with deionized water. Filter with a membrane filter.

2.4.2. Solvent B: Acetonitrile

2.5. Internal standard solution preparation

Transfer about 2 mg of NTTP-d_4 internal reference standard accurately weighed into a 10 mL volumetric flask, dissolve and dilute to volume with methanol as the internal standard stock solution. Upon use, dilute the internal standard stock solution with methanol to 5 ng/mL as the internal standard solution.

2.6. Standard solution preparation

Transfer about 5 mg of NTTP reference standard accurately weighed into a 10 mL volumetric flask, dissolve and dilute to volume with methanol as the standard stock solution. Upon use, mix appropriate volume of the standard stock solution and the internal standard solution, and dilute with methanol to 0.5-20 ng/mL (containing 0.5 ng/mL internal standard) as the standard solutions.

2.7. Standard calibration curve establishment

Accurately inject 5 μL of the standard solution into LC-MS/MS separately, and operate according to the following conditions. Establish the standard calibration curve of NTTP by the ratios of the peak area of NTTP to that of the internal standard vs. the concentrations of NTTP.

LC-MS/MS operating conditions(note):

Column: Hypersil GOLD C8, 3.0 μm, 4.6 mm i.d. × 10 cm.

Column temperature: 30°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows.

Time (min)	A (%)	B (%)
0.0 → 1.0	70 → 70	30 → 30
1.0 → 6.0	70 → 60	30 → 40
6.0 → 6.1	60 → 5	40 → 95
6.1 → 7.0	5 → 5	95 → 95
7.0 → 7.1	5 → 70	95 → 30
7.1 → 10.0	70 → 70	30 → 30

Flow rate: 0.5 mL/min.

Inject volume: 5 μL.

Ion spray voltage: 4.0 kV.
Ionization mode: ESI^+.
Ion source temperature: 500°C.
Nebulizer gas, Gas 1: 50 psi.
Heated gas, Gas 2: 70 psi.
Curtain gas: 40 psi.
Collision gas: high.
Detection mode: multiple reaction monitoring (MRM). Selected ion pair, declustering potential and collision energy are as follows.

Analyte	Ion pair Precursor ion (*m/z*) > Product ion (*m/z*)	Declustering potential (V)	Collision energy (eV)
NTTP	222 > 192*	30	24
	222 > 165	30	30
NTTP-d_4 (I.S.)	226 > 196	30	20

* Quantitative ion pair

Note: 1. If a divert valve is available, the direction of the mobile phase can be diverted as follows.

Time (min)	Position
0.0 → 3.5	Waste
3.5 → 4.5	MS
4.5 → 10.0	Waste

2. All the parameters can be adjusted depending on the instruments used if the above conditions are not applicable.

2.8. Sample solution preparation

Transfer about 0.1 g of sample accurately weighed to a centrifuge tube, and add 1 mL of the internal standard solution and 9 mL of methanol. Mix well, sonicate for 5 min and centrifuge at 3000 ×g for 5 min. Filter the supernatant with a membrane filter, and take the filtrate as the sample solution.

2.9. Identification and quantification

Accurately inject 5 μL of sample solution and standard solution into LC-MS/MS separately, and operate according to the conditions in section 2.7.

Identify NTTP based on the retention time and the relative ion intensities(note). Calculate the amount of NTTP in the sample by the following formula:

$$\text{The amount of NTTP in the sample (μg/g)} = \frac{C \times V}{M} \times 10^{-3}$$

Where,

C: the concentration of NTTP in the sample solution calculated by the standard calibration curve (ng/mL)

V: the final make-up volume of the sample (mL)

M: the weight of the sample (g)

Note: Relative ion intensities are calculated by peak areas of qualitative ions divided by peak areas of quantitative ions (≤ 100%). Maximum permitted tolerances of relative ion intensities by LC-MS/MS are as follows.

Relative ion intensity (%)	Tolerance (%)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
≤ 10	± 50

Remark

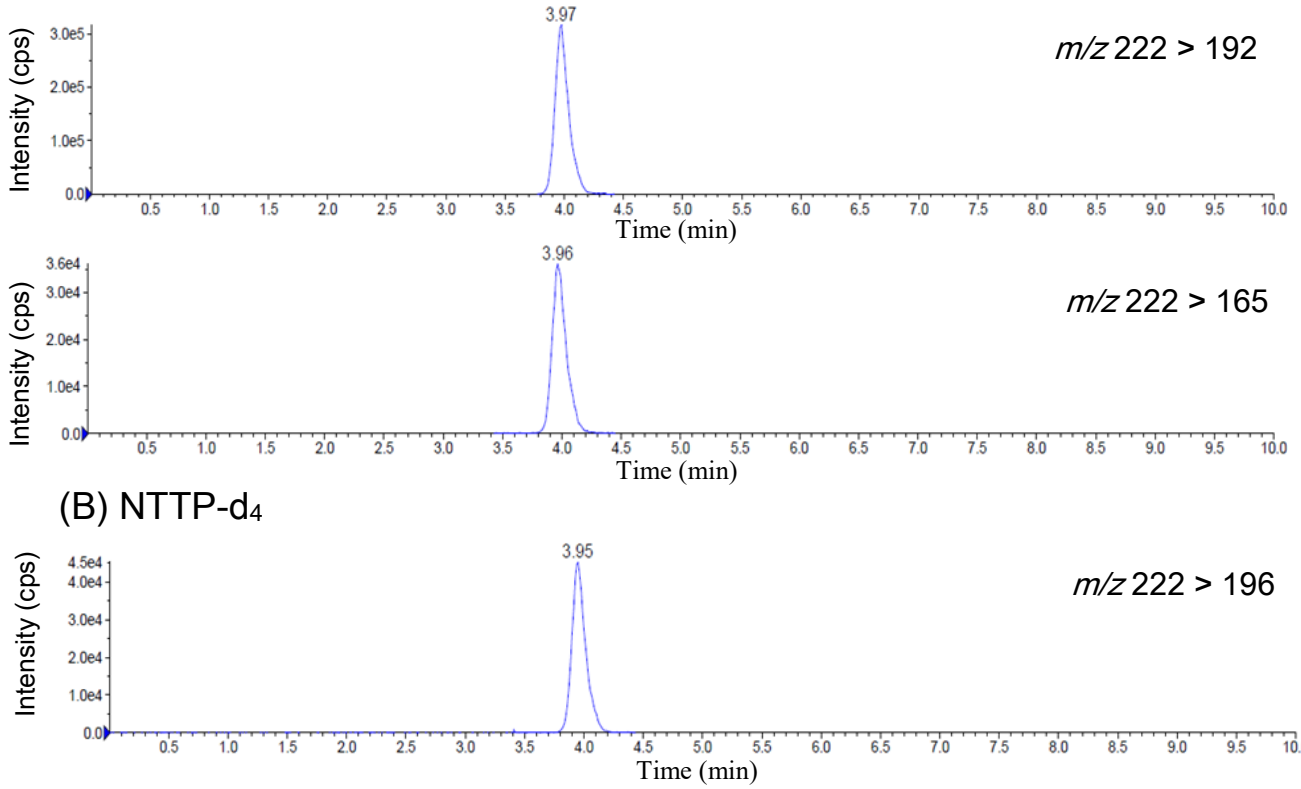
1. Limit of quantification (LOQ) for NTTP is 0.05 μg/g.
2. Further validation should be performed when interference compounds appear in samples.

Reference

1. Wang, Z., Hu, S., Wu, X., He, Z., Ke, C. and Hu, M. 2023. A highly sensitive LC-MS/MS method for the determination and quantification of a recently identified N-nitrosamine impurity in the sitagliptin phosphate monohydrate active pharmaceutical ingredient. Analytical Methods 15: 256-260.
2. Chittireddy, H. N. P. R., Kumar, J. V. S., Bhimireddy, A., Shaik, M. R., Khan, M., Adil, S. F., Khan, M. and Aldhuwayhi, F. N. 2022. Development and validation for quantification of 7-nitroso impurity in sitagliptin by ultraperformance liquid chromatography with triple quadrupole mass spectrometry. Molecules 27: 8581.

Reference chromatogram

(A) NTTP



(B) NTTP-d_4

Figure. The MRM chromatograms of NTTP standard (A) and NTTP-d_4 internal standard (B) analyzed by LC-MS/MS.