Determination of *N*-nitroso hydrochlorothiazide in various preparations

1 Objective and scope

The present method is used to quantify *N*-nitroso hydrochlorothiazide (NA-HCT) in various preparations by LC-MS/MS.

2 Principle of the method

Various preparations are extracted with methanol and water and the NA-HCT content is determined using an external calibration curve. Three series of preparations are made per sample, two of which are spiked with NA-HCT. The calculated content of the sample is correlated with the mean recovery of the two spiked preparations. If the mean recovery is below 50%, the content of the sample is determined using the standard addition method with 4 series of preparations (3 spiked).
NA-HCT degrades relatively quickly in solution at room temperature. All solutions must therefore be stored in a refrigerator or autosampler.

3 Validation data

See *in situ* validation

4 Context

- Method development A-024068

5 Definitions and abbreviations

See ***OMCL Glossary***

Term / Abbreviation	Description	Additional information
MRM	Multiple Reaction Monitoring	Selective and sensitive MS measuring mode
API	Active Pharmaceutical Ingredient	

6 Additional documents

For specific instructions, see ***BPM / Procedures / Tools*** or ***LIMS / Methods***

None.

7 Special measures / Safety instructions

NA-HCT is potentially carcinogenic. Appropriate protective measures must be taken. NA-HCT is not stable in solution at room temperature. All reference solutions and sample preparations must be stored in a refrigerator.

8 Reference and control substances, test equipment, materials, chemicals and solutions

8.1 Reference material

Name	Content / purity	LIMS S-No.	Manufacturer/supplier/ article no. (or equivalent)
4-Nitroso hydrochlorothiazide	See AZ	S-4382	Clearsynth Labs Ltd.

8.2 Control substances

Not applicable.

8.3 Equipment and materials

Name	**LIMS** procedure documentation E: documentation of results S: documentation of substances used
LC-MS/MS (e.g. 0524A)	E

8.4 Chemicals

Name	S-No. LIMS	Manufacturer/supplier/art. no. (e.g.)
Acetonitrile	S-2058	AppliChem GmbH / Axon Lab AG / 221881.1611
Methanol	S-1712	AppliChem GmbH / Axon Lab AG / 221091.1612
Formic acid	S-2031	Merck / Merck / 253
Ammonium formate	S-2034	Sigma-Aldrich Chemie GmbH
Milli-Q Water	S-2206	OMCL

8.5 Solutions

Solution name	Procedure	Shelf life / storage temp.
NA-HCT-stock solution	Weigh approx. 1 mg of the solution into a 10 mL volumetric flask and dilute to 10 mL with MeOH.	7 days at -20°C
NA-HCT-Dil 1	Dilute 50 µL of the stock solution to 20 mL with a mixture of methanol and Milli-Q water (1:1).	12 h at 2-8°C
NA-HCT-Dil 2	Dilute 1000 µL of the Dil 1 solution to 10 mLwith a mixture of methanol and Milli-Q water (1:1).	12 h at 2-8°C
Diluent	Mix 25 mL MeOH with 25 mL Milli-Q water.	14 days at RT
Eluent A	Add 315 mg ammonium formate to a 500 mL screw top bottle (e.g. from Schott), dissolve with 500 mL Milli-Q water and mix with 1.0 mL formic acid.	14 days at RT

To rule out errors in the weighing or dilution of the solution, the stock and Dil 1 solutions are each prepared in duplicate (A/B). As a control, a solution of the calibration line (5 ppm level) is prepared from the Dil 1 B solution and compared with the calibration line. The calculated concentration of the sample must not deviate by more than ±5% from the target concentration.

9 Procedure

A mixed sample is prepared from approx. 3 - 5 tablets. Weigh out a quantity of the mixed sample corresponding to 3 mg of active ingredient. However, the maximum quantity of medicinal product to be used is 150 mg. Three preparations are made per sample, two of which are spiked with NA-HCT (1 ppm / 2 ppm). As NA-HCT is not stable at room temperature, the final solutions must be placed in the autosampler/refrigerator as quickly as possible.

The quantification is carried out using an external calibration curve, whereby the calculated value is corrected with the mean recovery rate. If the mean recovery is below 50%, the content must be determined using the standard addition method with 4 preparations (3 spiked preparations, at 33%, 66% or 100% of the concentration determined with the external calibration, corrected with recovery).

The weighted samples are mixed with 500 µL of previously cooled methanol and the spiking material/solution and briefly vortexed. The resulting solution is then made up to 1000 µL with previously cooled Milli-Q water. The samples are vortexed again and extracted for 5 min on the rotary shaker. The samples are then centrifuged briefly (1-2 min) at approx. 20,000 g. An aliquot of the clear supernatant is analysed.

At the beginning of a sequence, calibration solutions (blank spike 0.75 ng/mL - 30 ng/mL) are analysed. After measuring each sample (3 injections), a blank extract is injected. After 12 sample injections (4 samples) and at the end of the sequence, a 15 ng/mL blank spike reference standard is measured. A blank is injected before and after the reference standard. Due to the instability of NA-HCT, a sequence should contain a maximum of 8 samples.

LC system

Column	Kinetex XB-C18, 2.1 x 100 mm, 1.7 µm (S-1668-690)		
Mobile phase A	Ammonium formate buffer pH 3.0		
Mobile phase B	Acetonitrile		
Autosampler temperature	5°C		
Column temperature	40°C		
Injection volume	5 µL		
Flow rate	0.3 mL/min		
Gradient	RT/ min	%A	%B
	0	90	10
	1.0	90	10
	10	80	20
	12	0	100
	14	0	100
	14.1	90	10
	17	90	10
UV Detection (optional)	220 nm, 4 nm bandwidth		

MS settings

Source	ESI / negative
Scan type	MRM
MRM detection window	Unscheduled
Curtain gas (CUR)	30
Collision Gas (CAD)	Medium
Temperature (TEM)	500
Ion Source Gas 1 (GS1)	40
Ion Source Gas 2 (GS2)	40
Ion Spray Voltage [IS]	-4500
Declustering Potential (DP)	70
Entrance Potential (EP)	10
Collision Cell Exit Potential (CXP)	13

MRM transitions

ID	Q1	Q3	CE	Dwell time [msec]
NA-HCT_1 (Quant)	325	294	-29	250
NA-HCT_2 (Qual)	325	217	-24	250
NA-HCT_3 (Qual)	325	281	-16	250

Diverter valve

RT	Diverter valve
0.0	to waste
7.0	to MS
10.0	to waste

10 Evaluation and measurement uncertainty

10.1 Evaluation

To determine the NA-HCT content, the area of the unspiked sample is converted into the concentration using the calibration line (1/x weighting, 6-points). The calculated concentration is then corrected with the mean recovery of the two spiking experiments.

$$recovery\,\% = \frac{C_{sp} - \frac{C_0 \cdot M_{sp}}{M_0}}{C_s} \cdot 100$$

C_{sp} : calculated concentration of the spiked sample / ng/mL
C_0 : calculated concentration of the unspiked sample / ng/mL
M_{sp} : mass of the spiked sample / mg
M_0 : mass of the unspiked sample / mg
C_S : concentration of the added spike in the sample / ng/mL

$$C = \frac{C_0 \cdot 2 \cdot 100}{W_1 + W_2}$$

C : Content in ng/mL
C_0 : Calculated concentration of the unspiked sample / ng/mL
W_1 : Recovery from spiking experiment 1
W_2 : Recovery from spiking experiment 2

If the average recovery ((W_1 + W_2)/2) is below 50%, the content of the sample must be determined using the standard addition method.

10.2 Measurement uncertainty

Calculated as part of the *in situ* validation.

11 Documentation and protocols

The requirements for documentation are given in the corresponding operating instructions on this topic. If necessary, specific requirements for documentation of process variable parameters are listed below.

Laboratory orders

No specific documentation is required for process variables.

12 Quality control

For quality control purposes, SST criteria are defined for the test series as well as for each sample.

SST criteria for analytical test series

Parameters	Criterion
Linearity Blank spikes (start of the sequence)	r ≥ 0.995

SST criteria for Sample

Parameters	Criterion
Mean recovery	≥ 50%
Correlation Standard addition (if performed)	r ≥ 0.995
Qualifier ratios	± 25%

13 Document history

Version no:	**Modification date/approval:**	**Change from previous version:**
1		Creation