Application Note

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Quantitation of N-Nitroso Hydrochlorothiazide in Losartan-Hydrochlorothiazide Tablets

Using the Agilent 6495D Triple Quadrupole LC/MS/MS System

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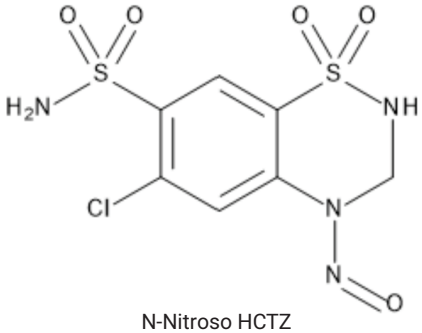
Abstract

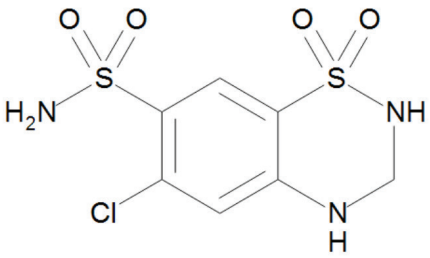
Losartan and hydrochlorothiazide (HCTZ) tablets combine an angiotensin II receptor blocker (ARB) and a thiazide diuretic to manage high blood pressure. This application note presents a sensitive and reproducible multiple reaction monitoring (MRM) method for quantifying N-nitroso HCTZ, a nitrosamine drug substance related impurity (NDSRI) in 100 mg losartan and 25 mg HCTZ tablets using an Agilent 6495D LC/MS/MS system. The method achieved a limit of detection (LOD) of 5 pg/mL and a limit of quantification (LOQ) of 10 pg/mL, with linearity from 10 pg/mL to 10 ng/mL (R^2 = 0.9951). Chromatographic separation was effective, and the method showed a signal-to-noise ratio (S/N) of 121:1 at the LOQ level. Spike recovery was 114.35% in placebo and 93.85% in tablets, with a percent relative standard deviation (%RSD) of 2.7% for seven replicate injections, confirming high reproducibility.

Introduction

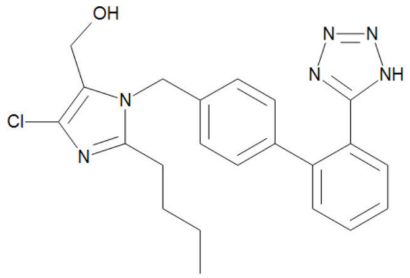
HCTZ tablets are a combination medicine used to manage high blood pressure. This formulation includes two active pharmaceutical ingredients (APIs), losartan and HCTZ, where losartan acts as an angiotensin II receptor blocker (ARB) and HCTZ as a thiazide diuretic. ARBs work by blocking the action of angiotensin II, which is a substance in the body that causes blood vessels to tighten. Reduced activity of ARB results in relaxing the blood vessels, thereby lowering blood pressure.



N-Nitroso HCTZ



HCTZ



Losartan

Figure 1. Chemical structures for N-nitroso HCTZ, HCTZ, and losartan.

HCTZ helps to reduce blood pressure by eliminating excess salt and water from the body through increased urine production. This combination is particularly effective for patients who require multiple medications to control their blood pressure, offering the convenience of a single tablet.

N-nitroso HCTZ is an NDSRI related to HCTZ and categorized as Class-4 in the potency categorization recommended by the U.S. FDA. The allowable intake maximum of N-nitroso HCTZ has been defined as 1,500 ng/day. Considering 25 mg HCTZ API in the formulation under study, the specification limit of N-nitroso HCTZ has been calculated to be 6 ppb for 0.1 mg/mL test concentration, which was used in this study.

Experimental

Materials

Calibration samples were prepared using N-nitroso HCTZ analytical standard material provided by an Indian pharmaceutical company. Formulation tablets, placebo, and the APIs for both drugs were also provided by same pharmaceutical company.

The following chemicals and reagents were also used: ammonium fluoride (MS grade, Fluka, Honeywell, Muskegon, Michigan, USA), acetonitrile (MS grade, Biosolve, Dieuze, France), and water (MS grade, Honeywell, Muskegon, Michigan, USA).

Equipment

Analysis was performed on a 1290 Infinity II LC system coupled with a 6495D Triple Quadrupole LC/MS/MS system equipped with the following modules:

- Agilent 1290 Infinity II high-speed pump (part number G7120A)
- Agilent 1290 Infinity II multisampler (part number G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (part number G7116B)
- Agilent 1290 Infinity II diode array detector (part number G7117A).

Method

The general LC/MS chromatography conditions are provided in Tables 1 to 3.

Table 1. Chromatography conditions.

Parameter	Value				
Column	Agilent ZORBAX SB C18, 4.6 × 150 mm, 5 µm (p/n 883975-902)				
Mobile Phase A	1 mM Ammonium fluoride in water				
Mobile Phase B	Acetonitrile				
Flow Rate	0.3 mL/min				
Injection Volume	20 µL				
Sample Cooler Temperature	8 °C				
Column Temperature	40 °C				
Needle Wash	Acetonitrile:water (90:10)				
Diluent	Methanol:water (1:1)				
Elution	Gradient				
Acquisition Time	28 minutes				
Gradient Program	Time (min)	%A	%B	Flow (mL/min)	Max. pressure limit (bar)
	0	90	10	0.3	1,300
	0.5	90	10	–	–
	8	50	50	–	–
	18	2	98	–	–
	22	2	98	–	–
	22.1	90	10	–	–
	28	90	10	–	–

Table 2. MS acquisition parameters.

Compound	Precursor	Product Ion (*m/z*)	Fragmentor Voltage (V)	Collision Energy (CE; V)	Polarity
N-nitroso HCTZ	325.0	293.9	166	20	Negative

Table 3. MS source parameters.

Parameter	Value
Ionization Source	Agilent Jet Stream Technology ion source (AJS) (p/n G1958B)
Gas Temperature	275 °C
Gas Flow	11 L/min
Nebulizer	35 psi
Sheath Gas	325 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	2,500 V
Nozzle Voltage	500 V
Gain	10.0

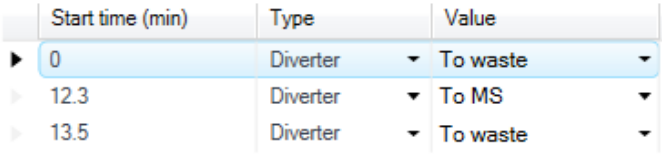


Figure 2. Diverter valve settings.

Preparation of standard and samples

Preparation of concentration linearity: A standard stock solution of 100 µg/mL was prepared by dissolving 2.5 mg of analytical standard using diluent and making up the volume in a 25 mL volumetric flask. Then, 1 mL of the stock solution was diluted to 100 mL to prepare a 1 ppm working standard solution.

A 10-point set of concentration linearity standards was prepared by serial dilution of the working standard solution.

Preparation of test samples: Five formulation tablets were accurately weighed and the average weight was determined. The tablets were then crushed to powder and mixed well using a pestle and mortar. A 0.1 mg/mL HCTZ content equivalent of the tablet powder was transferred to a 15 mL centrifuge tube and 10 mL of diluent were added. The tube was then vortexed for one minute followed by rotational shaking for 20 minutes using a Rotospin (Tarsons, Kolkata, India). Then, the solution was centrifuged at 9,500 rpm for 10 minutes and the supernatant was transferred to an HPLC vial for analysis. The same process was followed to prepare the placebo solution.

Two spiked samples were also prepared using same method by spiking LOQ-level (10 pg/mL) N-nitroso HCTZ into both placebo and tablet. Individual API solutions were also prepared at a concentration of 0.1 mg/mL.

Data acquisition and data analysis

Data were acquired using Agilent MassHunter Data Acquisition software (version 12.0). Chromatograms were viewed through MassHunter Qualitative Analysis software (version 12.0). Quantitation of each batch was carried out using MassHunter Quantitative Analysis software (version 12.1). Validation parameters (linearity, reproducibility, recovery, specificity, and sensitivity in terms of LOQ) were characterized to ensure good method performance. Accuracies for calibration points were within ± 20%. No manual integration was needed.

Results and discussion

The separation between the impurity and both APIs is shown in Figure 3.

The chromatogram clearly shows sufficient separation between the impurity and both APIs. To avoid further interference of the APIs on impurity response, the diverter program was used to automatically send the LC flow to waste during the elution of high concentration APIs, then divert it back to MS for the impurity.

Figure 4 shows the responses and S/N at LOD and LOQ levels, which were 5 and 10 pg/mL, respectively. A 10-point calibration curve was drawn with concentration linearity from 10 pg/mL to 10 ng/mL (Figure 5). The R^2 value for the calibration was found to be 0.995, which is well within the acceptable range.

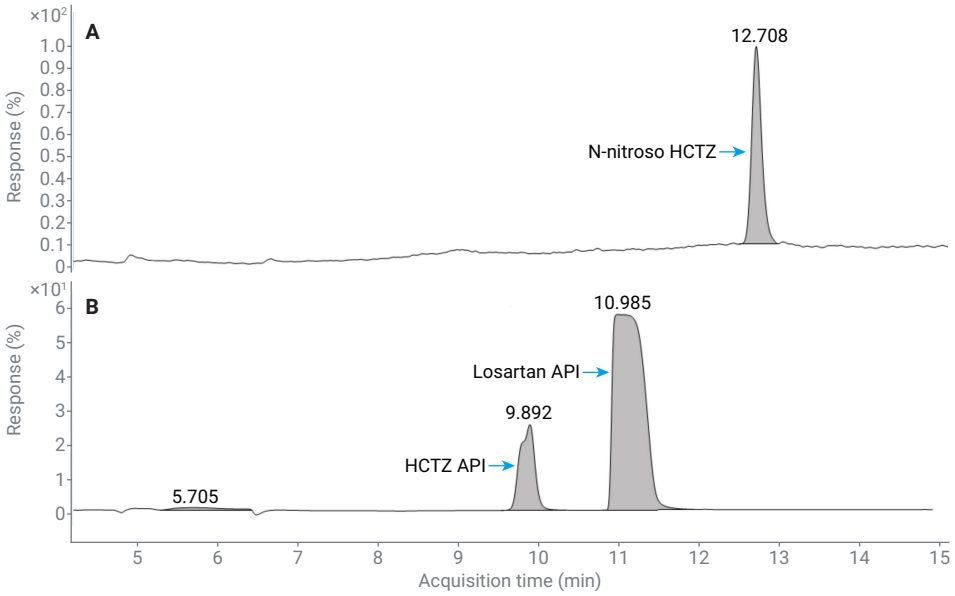


Figure 3. LC chromatograms showing resolution of (A) impurity and (B) APIs.

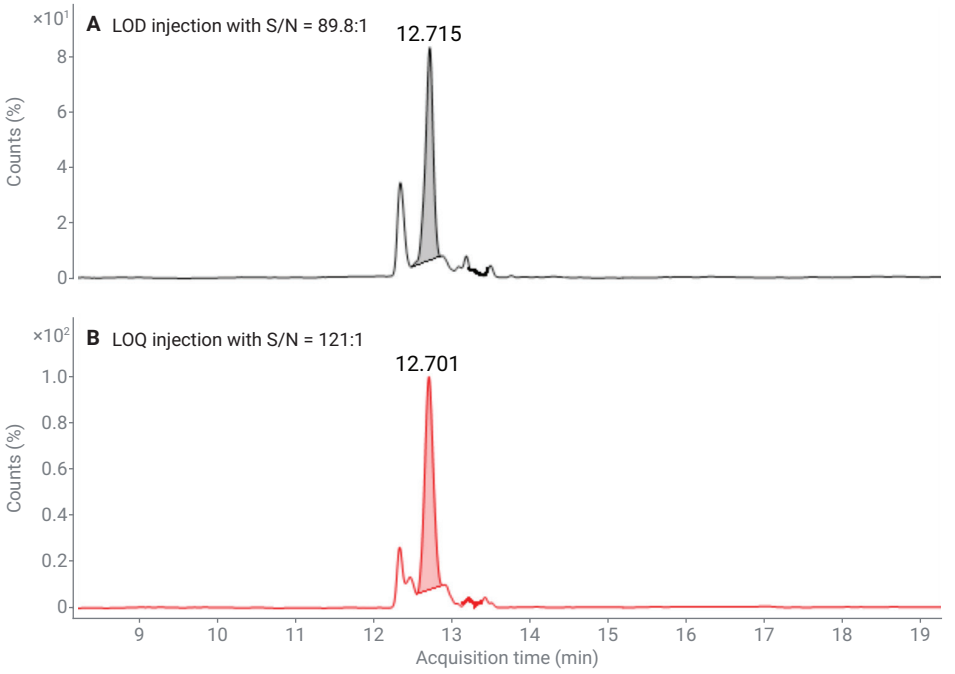


Figure 4. Chromatograms showing S/N at (A) LOD and (B) LOQ levels.

The tablet, API, and placebo solutions were injected as samples, and spiked placebo and tablet solutions were injected as QCs (with known spiked concentration of 10 pg/mL) along with the calibration standards. All the points of the calibration and QCs were found to be within the acceptable accuracy range of 80 to 120% (Figure 6).

The quant batch shows no impurity content in the placebo; the average absolute concentration of same in the formulation was also below LOQ level.

The recovery of the spiked samples was calculated by comparing the area of the neat standard at the LOQ level and the average area in the spiked samples using Equation 1.

Equation 1.

$$\frac{(\text{spiked area} - \text{unspiked area})}{\text{standard area}} \times 100$$

The results are shown in Table 4.

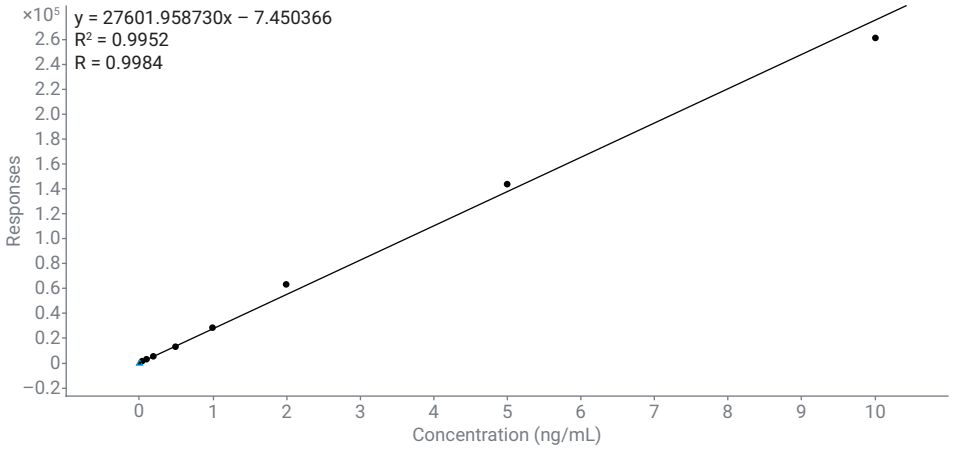


Figure 5. 10-point calibration curve from 10 pg/mL to 10 ng/mL (R^2 = 0.9952).

	Sample			NHCTZ Results			
	Name	Type	Level	RT	Resp.	Final Conc.	Accuracy
	BLANK	Blank		12.715	7	0.0005	
	0.01PPB	Cal	1	12.701	260	0.0097	96.9
	0.02PPB	Cal	2	12.701	446	0.0164	82.2
	0.05PPB	Cal	3	12.688	1260	0.0459	91.8
	0.1PPB	Cal	4	12.701	3269	0.1187	118.7
	0.2PPB	Cal	5	12.708	5369	0.1948	97.4
	0.5PPB	Cal	6	12.701	13154	0.4768	95.4
	1PPB	Cal	7	12.708	28621	1.0372	103.7
	2PPB	Cal	8	12.708	63430	2.2983	114.9
	5PPB	Cal	9	12.715	143860	5.2122	104.2
	10PPB	Cal	10	12.715	261381	9.4699	94.7
	BLANK	Blank		12.708	6	0.0005	
	API	Sample		12.735	10	0.0006	
	API	Sample		12.667	23	0.0011	
	API	Sample		12.688	17	0.0009	
	BLANK	Blank		12.735	3	0.0004	
	PLACEBO	Sample		12.653	3	0.0004	
	PLACEBO	Sample		12.619	6	0.0005	
	PLACEBO	Sample		12.824	7	0.0005	
	BLANK	Blank		12.626	9	0.0006	
	BLANK	Blank		12.954	6	0.0005	
	TAB	Sample		12.688	45	0.0019	
	TAB	Sample		12.653	51	0.0021	
	TAB	Sample		12.660	52	0.0021	
	BLANK	Blank		12.749	2	0.0003	
	PL-10	QC	1	12.708	299	0.0111	110.9
	PL-10	QC	1	12.695	307	0.0114	114.0
	PL-10	QC	1	12.708	302	0.0112	111.9
	BLANK	Blank		12.688	1	0.0003	
	BLANK	Blank		12.729	4	0.0004	
	TAB-10	QC	1	12.688	276	0.0103	102.6
	TAB-10	QC	1	12.688	304	0.0113	112.9
	TAB-10	QC	1	12.695	300	0.0112	111.5
	BLANK	Blank		12.681	6	0.0005	

← API injections in triplicate
← Placebo injections in triplicate
← Tablet injections in triplicate
← Spiked pacebo injections in triplicate
← Spiked tablet injections in triplicate

Figure 6. Analytical results for tablet, API, and placebo solutions.

Table 4. Calculated sample recoveries.

Sample ID	Average Area	Spiked Amount (ng/mL)	Area for the Neat Standard of Spike Concentration	Recovery (%)
Placebo	5.3	–	–	114.35
Placebo Spike	302.6	0.01	260	
Tablet	49.3	–	–	93.85
Tablet Spike	293.3	0.01	260	

Conclusion

A highly sensitive and robust MRM method was developed for the quantitation of N-nitroso HCTZ in 100 mg losartan and 25 mg HCTZ tablet formulation using an Agilent 6495D Triple Quadrupole LC/MS/MS system. The chromatographic method developed provided good separation between the analyte and the API. The method showed an LOD and LOQ of 5 pg/mL (0.05 ppm with respect to test) and 10 pg/mL (0.1 ppm with respect to test), respectively. The minimum signal-to-noise ratio at the LOQ level was found to be 121:1 (RMS). The three orders of calibration curve from 10 pg/mL to 10 ng/mL was found to be linear, with 1/x weighting. R and R^2 values were 0.9984 and 0.9952, respectively. Spike recovery analysis showed the efficiency of sample extraction with an average recovery percentage of 114.35% in the placebo and 93.85% in the tablet at the LOQ spiking level. The method was found to be highly reproducible at the LOQ level; the %RSD value for the area response of seven replicate injections was 2.7%.

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