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HPLC-CAD impurity profiling of carbocisteine using SCX-RP mixed-mode chromatography

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Goal

To analyze the non-proteinogenic amino-acid carbocisteine and its impurities by means of HPLC Charged Aerosol Detection (CAD) and to obtain improved sensitivity for the impurities when compared to the existing method.

Application benefits

- Modernized and translated UHPLC-CAD method
- LOQs reduced by average of 44% across all impurities

Introduction

Carbocisteine is a non-proteinogenic amino acid that is used in the treatment of acute and chronic respiratory diseases that require mucolytic agents.[1] As an amino acid, and thus with amino acids and similar structures in the impurity profile, it is challenging to establish a proper chromatographic procedure due to the substances being very similar in their physicochemical properties. The lack of a chromophore is often overcome with methods such as ninhydrin or ortho phthalaldehyde (OPA) derivatization, but this leaves the detection blind to other impurities such as organic acids or other substances that do not react with the derivatization agent such as cyclization products. The European Pharmacopoeia still sometimes uses simple TLC tests with ninhydrin-derivatization that only semi-quantitatively assess the impurity content relative to a reference spot.[2] Reliable and quantitative HPLC separation and detection methods are desirable. Therefore, charged aerosol detection (CAD) is a more convenient and direct approach for all non-volatile impurities making the Thermo Scientific™ Vanquish™ Charged Aerosol Detector a well-suited instrument.

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When used as a drug in humans, with a maximum daily dose of >2 g, the ICH guideline Q3A(R2) requires a reporting threshold for every impurity of 0.03% (m/m).[3] It is desirable to have reliable detection with a LOQ better than this value. The already published method lacked a sufficient LOQ for cystine, with only 0.09%, so an improvement of the sensitivity is the goal.

In this application, a method formerly published by Wahl and Holzgrabe[4] was slightly modified to result in a mobile phase of 18% acetonitrile and 10 mM trifluoroacetic acid (TFA). This method was run on a strong cation exchange reversed-phase (SCX-RP) mixed-mode column and the newest generation Vanquish Charged Aerosol Detector. The requirements according to the ICH guidelines of a reporting threshold of 0.03% for each impurity were met and LOQs of 0.02% or lower were obtained. These LOQs are far lower than LOQs of the original method on the Thermo Scientific™ Corona™ Charged Aerosol Detector (Corona CAD), which only reached 0.09% for cystine. Linear models of calibration curves for all impurities over a range of 0.05-0.25% of the assay's concentration yielded $R^2 > 0.995$. When analyzing the same batches as in the experiments from Wahl and Holzgrabe, batches that formerly could only be labeled with "not detected" for their cystine content could now be assigned with a low percentage value.

Experimental

Recommended consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Scientific™ Optima™ UHPLC-MS grade acetonitrile (P/N A956-1)
- Fisher Scientific™ Optima™ LC-MS grade trifluoroacetic acid (TFA) (P/N A116-50)
- Fisher Scientific™ Analytical grade ammonia (P/N A/3295/PB05)
- Thermo Scientific™ Chromacol™ vial, clear 1.5 mL kit with septa and cap (P/N 2-SVWGKST-CPK)

Mobile phase preparation

Only high purity solvents are to be used with the Vanquish CAD because it will detect semi and non volatile contaminants present in the mobile phase and samples.

Prepare a 0.1 M TFA solution by adding 5.70 g of TFA to about 300 mL of 18.2 MΩ·cm resistivity deionized water, and then bring the volume up to 500 mL. Combine 180 mL of acetonitrile and 100 mL of the 0.1 M TFA solution in a 1000 mL volumetric flask and bring up to volume with water.

Sample preparation

Carbocisteine and its impurities (Figure 1) are polar and thus water-soluble with certain limitations as described below.

Stock solutions of the impurities were prepared at a concentration of 0.25 mg/mL by exactly weighing 2.5 mg and diluting with water to 10.0 mL. To overcome solubility issues, an addition of 3% of concentrated ammonia solution to the stock solution of cystine was made. For cystine the stock solution should be prepared at a concentration of 0.1 mg/mL for reasons of solubility.

All sample solutions must be freshly prepared by exactly weighing 50 mg of carbocisteine and diluting with water to 10.0 mL after the addition of 300 µL of concentrated ammonia solution.

The impurity stock solutions can be stored at 2 °C to 8 °C and diluted daily. Setting the autosampler temperature to 8 °C was found to be sufficient to use the vials in the rack on multiple days.

Method optimization summary

The initial method utilizes an acetonitrile content of 12% (v/v) with 0.1 mM TFA and detection with the Corona CAD with a filter setting of "high". Evaporation temperature could not be changed on that instrument.

The original method was used with a systematic variation of evaporation temperature settings of the CAD ranging from 25 °C to 60 °C. The signal-to-noise ratio for a cystine solution containing 0.0025 mg/mL was observed. It was calculated by the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software using the peak-to-peak noise in a fixed interval over the last two minutes of the run.

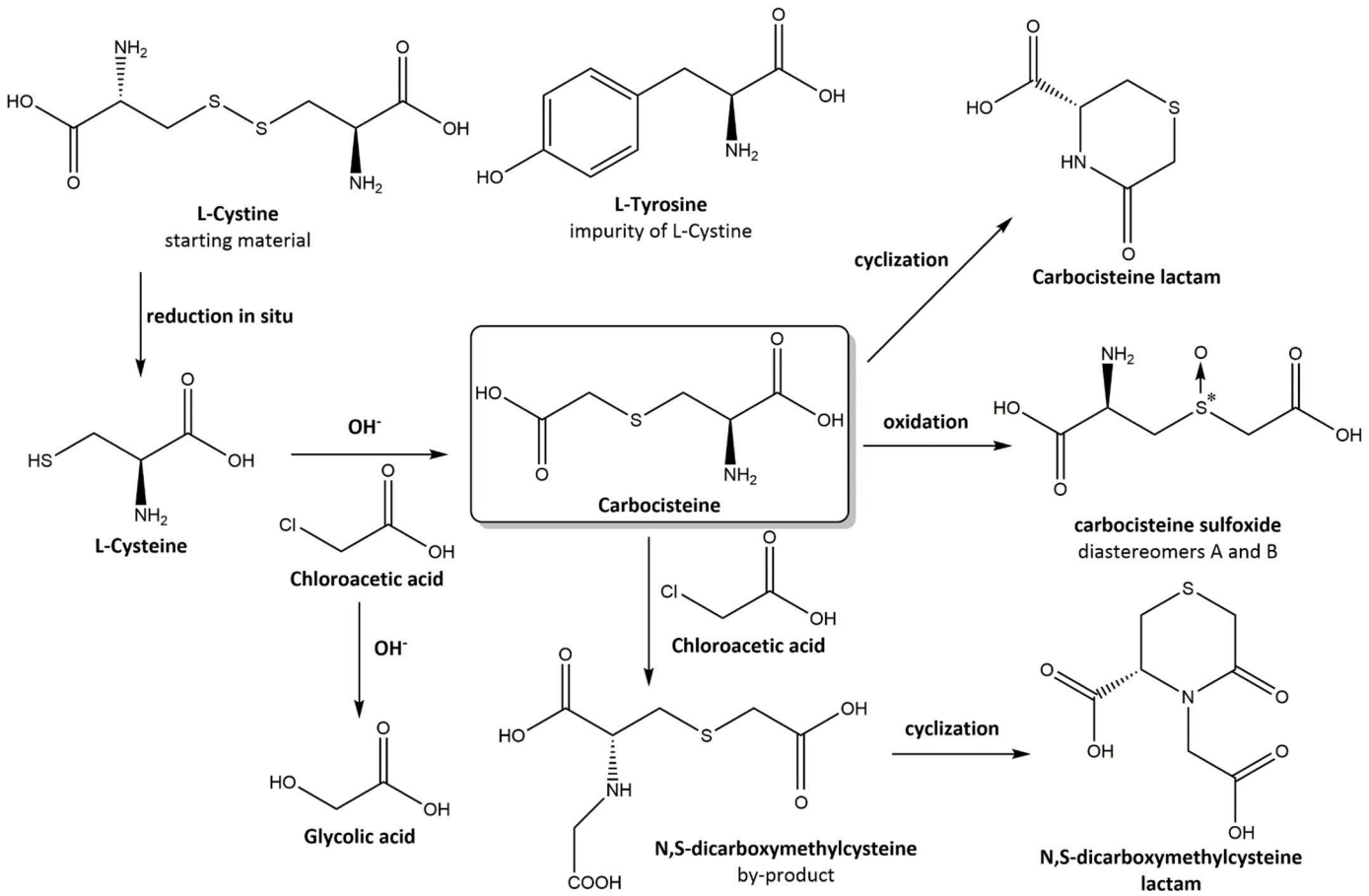

Figure 1. Structures and origin of carbocisteine and its impurities[1]

This concentration equaled 0.05% of the assay's concentration. The signal-to-noise-ratio of this 0.05% cystine solution obtained using the original method with the Vanquish CAD was >30 (see Figure 3) while it was below the LOQ with the older CAD. These preliminary experiments with the original mobile phase showed that the performance of the Vanquish CAD is superior to the earlier generation Corona CAD from Thermo Fisher Scientific.

The best result was achieved with an evaporation temperature of 50 °C and a filter setting of 10 s, which corresponds to the "high" setting on the Corona CAD.

When injecting an impurity mixture to check the separation of the method, an interesting observation was found with the late eluting analytes, tyrosine and cystine. On the one hand, they eluted too close to each other and on the other hand, they changed their elution order when injected with a carbocisteine sample instead of an impurity mixture or individually. The reason behind this was not further investigated because this phenomenon could be overcome by increasing the acetonitrile content.

Variations of TFA and acetonitrile content were examined. As already described in the original publication, the retention of the carbocisteine lactam and the elution order of cystine and tyrosine is governed by acetonitrile content. The TFA content affects peak shape and general retention for the two late eluting impurities. Increasing the acetonitrile content to 18% v/v instead of the initial 12% v/v showed the desired behavior of a good separation and tyrosine eluting before cystine. Additionally, sensitivity is increased with higher percentage of organic modifier.[5]

Instrumentation

Vanquish UHPLC system equipped with:

- System Base (P/N VH-S01-A)
- Vanquish Charged Aerosol Detector H (P/N VH-D20-A)
- Vanquish Binary Pump Flex (P/N VF-P10-A-01)
- Vanquish Split Sampler (P/N VF-A10-A)
- Vanquish Column Compartment H (P/N VH-C10-A)
- Vanquish Diode Array Detector F (P/N VF-D40-A)

Conditions

Column:	SIELC Primesep® 100; 250 × 4.6 mm, 5 µm
Mobile phase:	The mobile phase comprised 18/82 acetonitrile/ultrapure water (v/v) and 10 mM TFA.
Flow rate:	1.3 mL/min
Run time:	20 min
Column temp.:	20 °C
Injection volume:	20 µL
Vanquish CAD detector settings	
Evaporation temp.:	50 °C
Power function:	1.00
Data collection rate:	10 Hz
Signal filter:	10 s

Data processing

Chromeleon CDS Version 7.2.6 was used for data acquisition and analysis.

Results and discussion

Separation of carbocisteine and its impurities

Separation of carbocisteine and its possible impurities (Figure 1) was achieved using a mixed-mode column with both hydrophobic and strong cation exchange functionalities, SIELC Primesep 100 column. Due to the combined retention mechanisms, neutral impurities, e.g. carbocisteine lactam, and the polar amino acids tyrosine and cystine could be separated within 20 minutes isocratically (Figure 2). The two peaks due to the carbocisteine sulfoxide diastereomers A and B were analyzed by the sum of their respective areas.

The mobile phase comprised 18/82 acetonitrile/ultrapure water (v/v) + 10 mM TFA. The amount of acetonitrile in the mobile phase mainly affected the separation of the early eluting impurity carbocisteine lactam and the late eluting impurity tyrosine; whereas the TFA concentration was crucial for the retention of the late eluting impurities tyrosine and cystine. The acetonitrile content was increased compared to the previous method because more acetonitrile had a positive impact on signal height and resolution was still acceptable. Furthermore, a reliable resolution and elution order between tyrosine and cystine was obtained. The TFA content was not changed from the old method since it offered the best compromise in peak shape and retention times of the late eluting impurities.

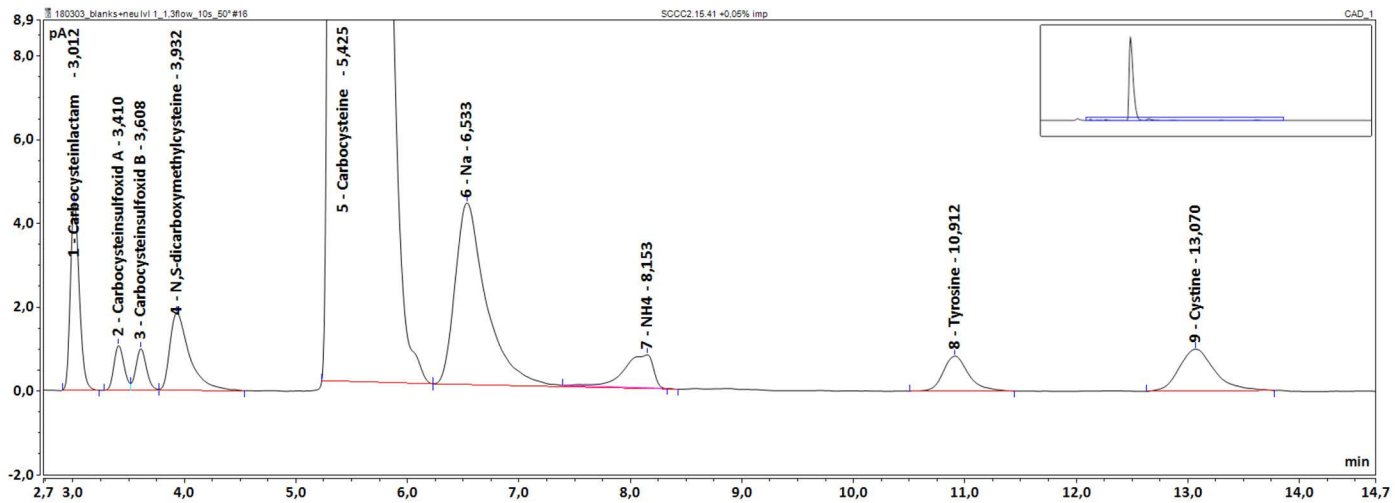

Figure 2. Batch sample of carbocisteine spiked with 0.05% of each impurity; transferred method

Linearity and limit of quantitation

Calibration curves for all detectable impurities were obtained by injecting five concentration levels covering the suspected range of impurity content in the samples (0.05%–0.25%). The coefficients of determination are shown in Table 1. Although the CAD generally is a non-linear detector, in the observed small concentration range an almost linear relation between analyte concentration and detector signal can be assumed.

The LOQ is crucial for this application because the relevant guidelines of the ICH and EDQM claim a reporting threshold of not more than 0.03% of each impurity. The analytical procedure's LOQ should not exceed that threshold, thus a LOQ of at least 0.03% of the test substance's concentration is highly desirable.

Compared to the old method using the Corona CAD as the detector, the Vanquish CAD showed improved sensitivity resulting in lower LOQs for all impurities except for carbocisteine sulfoxide (Table 1). The LOQ was obtained by the signal-to-noise (S/N) approach of ICH guideline Q2(R1) corresponding to an analyte's concentration that gives a S/N ratio of 10. For cystine, the S/N ratio was also determined at an acetonitrile content of 12% to enable comparison of the sensitivity of the new Vanquish CAD with the older model. A concentration of 0.05% cystine referred to the test substance carbocisteine at 5 mg/mL, thus corresponding to the qualification threshold of the ICH guidelines. This resulted in a S/N ratio of 30, demonstrating superior sensitivity of the Vanquish CAD model (Table 1 and Figure 3).

Table 1. Limits of quantitation and coefficients of determination

Analyte	R^2	LOQ [µg/mL]	LOQ [%]	LOQ Old Method [%]
Carbocisteine lactam	0.9983	0.70	0.01	0.02
Carbocisteinsulfoxide	0.9973	0.94	0.02	0.02
N,S-dicarboxymethyl cysteine	0.9990	0.97	0.02	0.04
Tyrosine	0.9990	1.14	0.02	0.03
Cystine	0.9995	0.69	0.01	0.09

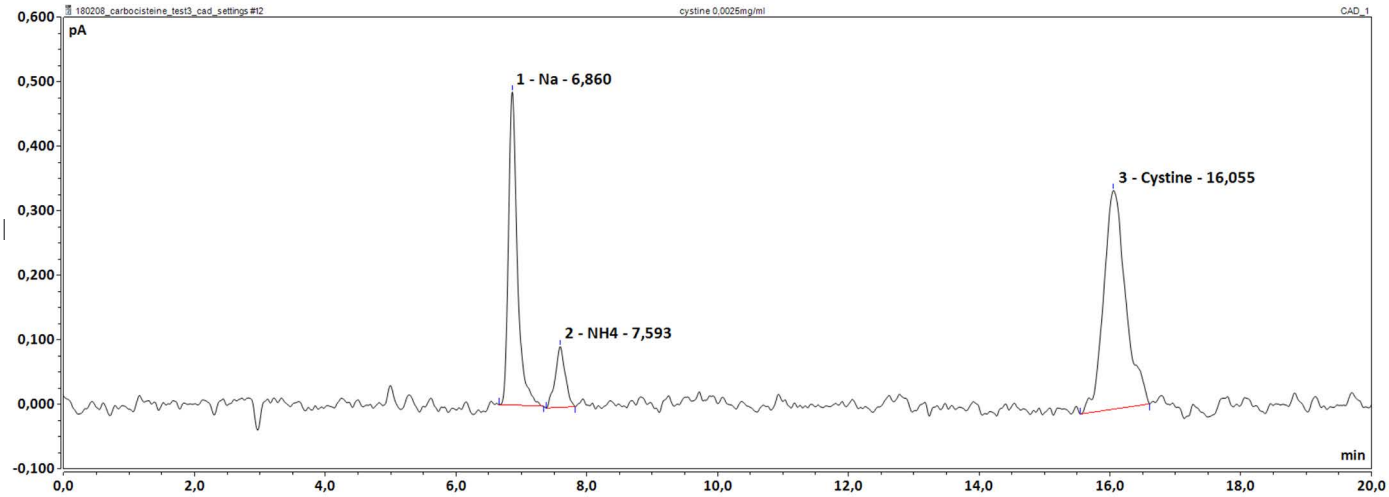

Figure 3. Cystine at 0.05% referred to the assay's concentration, run with 12% acetonitrile (v/v)

Accuracy and precision

Accuracy was assessed with spiked samples at three levels of impurity content (0.05%, 0.15%, and 0.25%). The spiked samples were injected repetitively (n=6) and the recovery of each impurity was calculated (Table 2). The average recovery rates were slightly increased at the 0.05% level. This can be accepted since a minor overestimation of impurities could be favorable around the reporting and qualification threshold with regard to drug safety.

Repeatability was investigated in terms of intra- and interday precision on a batch sample containing all specified impurities. The sample was injected repetitively (n=6) on day 1 and day 2 (Table 3). The RSD of each impurity was below 10%, indicating sufficient precision of the method.

Sample analysis

Eleven different batches of four manufacturers were analyzed using the described method. A specific impurity profile was observed for each manufacturer. Cystine, which was not always detectable by the old method, could be identified and estimated in every sample (Table 4). The transferred method on the Vanquish CAD shows superior sensitivity over the original method on the Corona CAD, leading to a more reliable assessment of low level impurities, especially for cystine. Due to the superior LOQs the regulatory requirements could be entirely met.

Table 2. Recovery rates at spiking level 0.05%, 0.15%, and 0.25%

Analyte	Recovery Rate (0.05%, n=6)	Recovery Rate (0.15%, n=6)	Recovery Rate (0.25%, n=6)
Carbocisteine lactam	111%	106%	105%
Carbocisteinsulfoxide	116%	99%	98%
N,S-dicarboxymethyl cysteine	112%	91%	95%
Tyrosine	119%	111%	108%
Cystine	111%	102%	101%

Table 3. Intra- and interday precision

Analyte	Repeatability Day 1 (RSD %, n=6)	Repeatability Day 2 (RSD %, n=6)	Interday Precision (RSD %, n=12)
Carbocisteine lactam	1.38	1.30	2.34
Carbocisteinsulfoxide	7.92	4.01	7.37
N,S-dicarboxymethyl cysteine	4.36	2.16	4.37
Tyrosine	9.16	7.08	8.19
Cystine	8.12	7.56	9.62

Table 4. Batch result comparison for cystine content, n.d. = not detectable

Batch	Cystine	Cystine Old Method
a1	0.333%	0.37%
a2	0.351%	0.41%
b1	0.039%	n.d.
b2	0.031%	n.d.
b3	0.040%	n.d.
c1	0.089%	0.09%

Conclusion

A method for the impurity profiling of the drug carbocisteine based on CAD detection was successfully transferred from the first-generation Corona CAD to the newest model, Vanquish CAD. The regulatory requirements that claim a reporting threshold of 0.03% impurity content could easily be met for most of the tested impurities, which was not the case before. The new approach offers superior sensitivity resulting in lower LOQs for most of the tested impurities.

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