Two-Dimensional Liquid Chromatography-Mass Spectrometry Analysis of Impurities in Amoxicillin

Abstract (Preface)

Amoxicillin (hydroxyampicillin), CAS No. 26787-78-0, is a widely used broad-spectrum β-lactam antibiotic of the penicillin class. It is unstable under high temperature, light, acid, alkali, and oxidation conditions. Clinical evidence shows that amoxicillin polymers can induce allergic reactions. Therefore, related substances generated during production and storage directly affect product quality and clinical safety.

In the *Chinese Pharmacopoeia* 2020 Edition, related substances of amoxicillin are analyzed by high-performance liquid chromatography with ultraviolet detection, using potassium dihydrogen phosphate solution and acetonitrile as mobile phases. Since non-volatile phosphate salts are incompatible with mass spectrometry, this study employs online column-switching two-dimensional liquid chromatography. Target impurity peaks from the first dimension are transferred to the second-dimensional column via valve switching for online desalting, followed by qualitative analysis using a mass-spectrometry-compatible mobile phase.

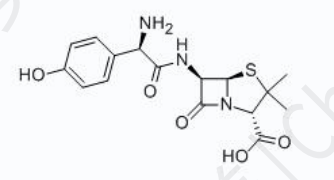


Figure 1 Chemical Structure of Amoxicillin (MW 365.4)

Instrument System

- Chromai two-dimensional liquid chromatography system: dual ternary pumps P60, auto-sampler A10C, column oven C10V2 with two two-position six-port valves, ultraviolet detector D10
- Chromai triple quadrupole mass spectrometer (TQ5000)

Figure 2 Chromai Liquid Chromatography-Mass Spectrometer (TQ5000)

Liquid Chromatography Conditions

- First-dimensional column: Lotus ACC4 4.6×250 mm, 5 μm
- Second-dimensional column: Lotus AQ-C18 4.6×250 mm, 5 μm
- First-dimensional mobile phase A: 0.05 mol/L potassium dihydrogen phosphate (adjusted to pH 5.0 with 2 mol/L KOH) : acetonitrile = 99:1
- First-dimensional mobile phase B: 0.05 mol/L potassium dihydrogen phosphate (adjusted to pH 5.0 with 2 mol/L KOH) : acetonitrile = 80:20
- Second-dimensional mobile phase A: 0.1% formic acid in water
- Second-dimensional mobile phase B: acetonitrile
- Column temperature: 35 °C
- Flow rate: 1.0 mL/min
- Loop volume: 1000 μL
- Injection volume: 50 μL
- Detection wavelength: 254 nm

Mass Spectrometry Parameters

- Ionization source: ESI
- Scan mode: Q1 full scan, positive mode m/z 200–1200; negative mode m/z 80–500
- Product ion scan: m/z 100–400, standard resolution, CE 10 V, CAD 8
- Ion source temperature: 550 ℃
- Auxiliary heater gas: 50 psi
- Nebulizer gas: 40 psi
- Curtain gas: 40 psi

Sample Pretreatment

Weigh 20.0 mg of amoxicillin, dissolve ultrasonically with first-dimensional mobile phase A, and dilute to 10.0 mL. Filter through a 0.22 μm membrane filter before analysis.

Results and Discussion

Retention times of amoxicillin impurities in 1D and 2D chromatography are listed in Table 1. Target peaks from the first dimension were collected in the loop and transferred to the second-dimensional column. Online desalting was performed from 0 to 10 min (diverted to waste), and MS analysis started at 10.1 min.

Table 1 Retention Times and Valve Switching Times

1D Peak No.	2	5	6	7	8	9
1D RT / min	7.742	25.842	28.905	30.946	33.545	34.259
2D RT / min	12.345	12.427	12.544	14.043	13.662	13.767

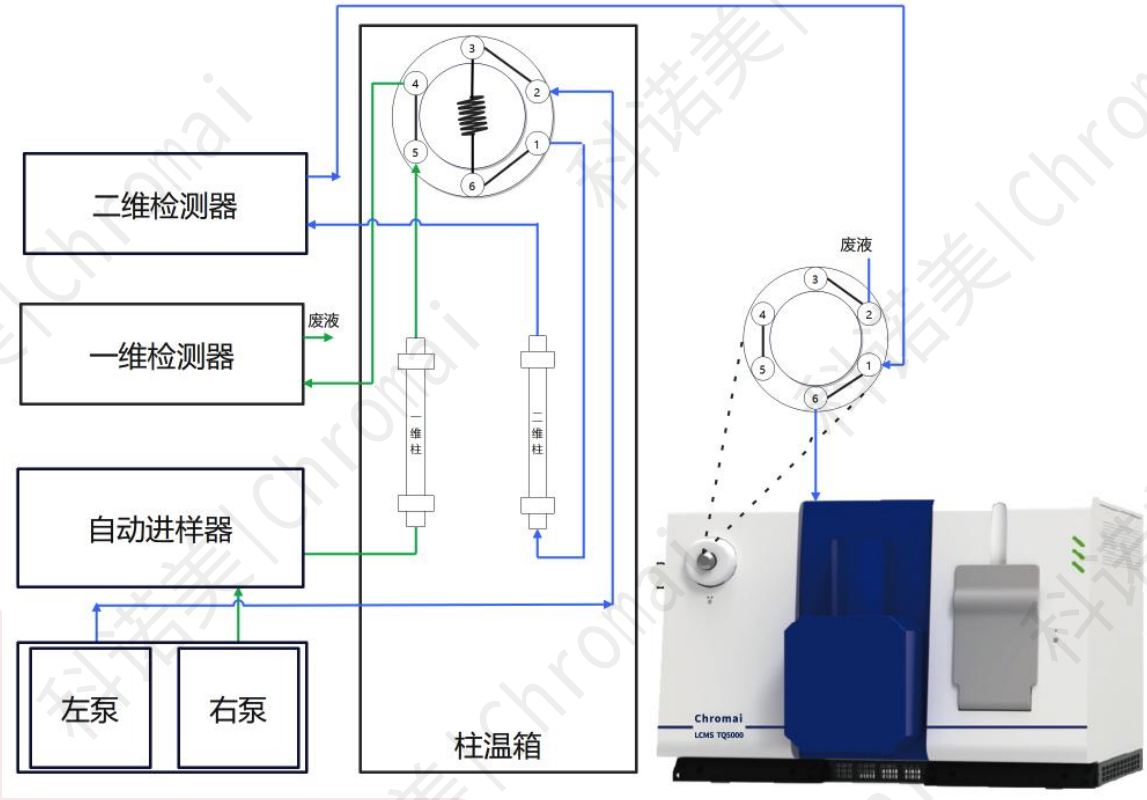
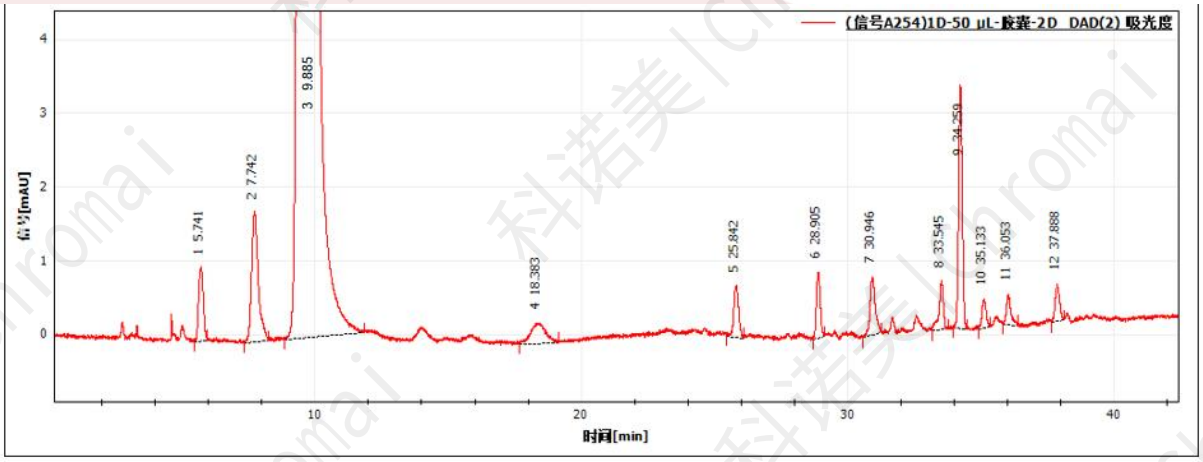


Figure 3 Schematic Diagram of Two-Dimensional Liquid Chromatography-Mass Spectrometry

In the first-dimensional chromatogram, peaks 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12 are impurities; peak 3 is the main amoxicillin peak.



Parent ions and characteristic fragment ions were obtained. Combined with literature, impurities were tentatively identified.

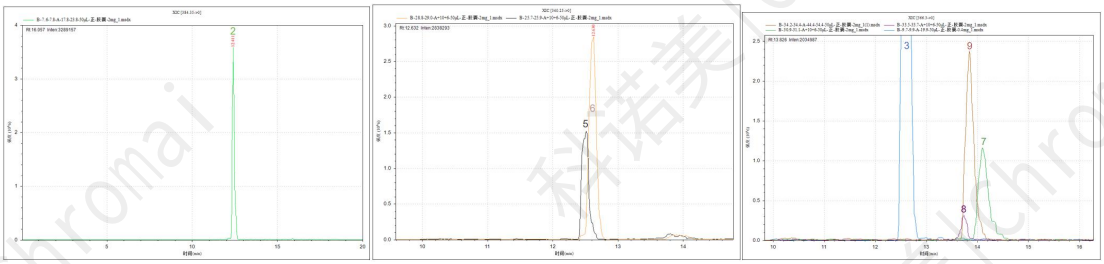


Figure 5 Extracted Ion Chromatograms of Each Numbered Impurity Peak (Extraction Deviation 0.5)

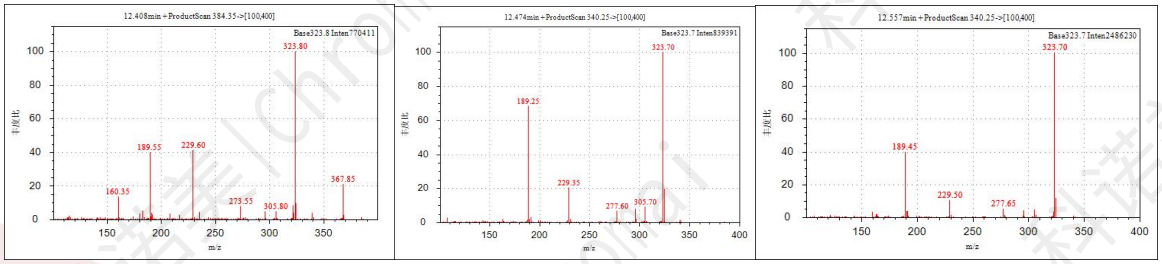


Figure 6 Product Ion Scan Characteristic Ion Spectrum of Peak 2 Figure 7 Product Ion Scan Characteristic Ion Spectrum of Peak 5 Figure 8 Product Ion Scan Characteristic Ion Spectrum of Peak 6

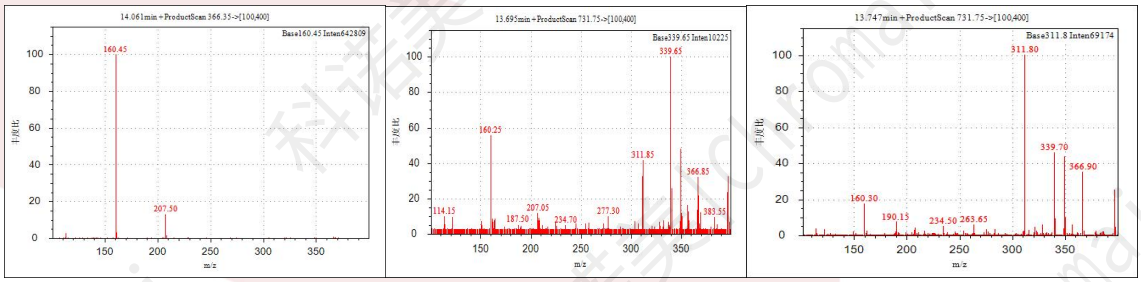


Figure 9 Product Ion Scan Characteristic Ion Spectrum of Peak 7 Figure 10 Product Ion Scan Characteristic Ion Spectrum of Peak 8 Figure 11 Product Ion Scan Characteristic Ion Spectrum of Peak 9

Table 2 Summary of Full Scan and Product Ion Scan Data

Peak No.	Positive full scan m/z	Product ion m/z	Negative full scan m/z
2	384.35	367.85, 323.80, 229.60, 189.55	382.25, 338.20
5	340.25	323.70, 229.35, 189.25	338.20
6	340.25	323.70, 229.50, 189.45	338.30
7	366.30	207.50, 160.45	364.25, 410.25
8	366.25, 731.50	396.95, 366.85, 339.65, 311.85, 160.25	729.35
9	366.45, 731.60	396.95, 366.90, 339.70, 311.80, 160.30	729.55

Impurity Identification

- Peak 2: amoxicilloic acid (5S,6R) or (5R,6R)
- Peaks 5 & 6: isomers, amoxilloic acid (5S) or (5R)
- Peak 7: isomer of amoxicillin, e.g., L-amoxicillin or piperazine-2′,5′-dione
- Peaks 8 & 9: amoxicillin dimer

Conclusion

A method using online column-switching two-dimensional LC-MS with Chromai dual ternary pumps was established for rapid qualitative analysis of six trace impurities in amoxicillin. Online desalting enables direct MS identification. The method supports structural elucidation and quality control of amoxicillin API.

References

[1] Nägele E, Moritz R. Structure elucidation of degradation products of the antibiotic amoxicillin with ion trap MS and accurate mass determination by ESI TOF. J Am Soc Mass Spectrom, 2005, 16(10): 1670–1676.[2] Valvo L, Ciranni E, Alimenti R, et al. Development of a simple liquid chromatographic method with UV and mass spectrometric detection for the separation of substances related to amoxicillin sodium. J Chromatogr A, 1998, 797(1–2): 311–316.