

Research Paper

Establishment and validation of the LC-MS/MS method for the determination of lincomycin in human blood: Application to an allergy case in forensic science

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ABSTRACT

An analytical method to quantify lincomycin in human blood samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been developed and validated. The selected method was based on a protein precipitation extraction (PPE) with methanol. Instrumental determination was carried out by LC-MS/MS, with quantification based on the internal standard method. Linearity for lincomycin was established in the concentration range of 5–100 ng/mL. The limit of detection (LOD) and limit of quantification (LOQ) were 0.2 and 1 ng/mL, respectively. Analyte recoveries were in the range of 72.70%–84.13% for spiked blood samples. The accuracies ranged between 92.82% and 100.40%, and the intraday and inter-day precisions ranged between 1.19% and 6.40%, respectively. The developed method was applied to an authentic allergy case of lincomycin. By testing the lincomycin content in the venous blood of the deceased and combined with the pathological test results, lincomycin acute allergy appeared to be the most likely cause of death. The acquired results confirm that the developed method is capable of identifying and quantifying lincomycin in human blood and can be suitable for the detection of allergy cases in clinical or forensic science.

1. Introduction

Lincomycin is a type of lincosamide antibiotic, with strong antibacterial ability against gram-positive aerobic bacteria, which also demonstrates activity against gram-positive or negative anaerobic bacteria.^{1,2} This antibiotic can irreversibly bind to a site on the 50S subunit of bacterial ribosomes to selectively inhibit protein synthesis by blocking the extension of the peptide chain, which belongs to a kind of growth period bacteriostatic agent.^{3,4}

As a common antibiotic, lincomycin is widely used worldwide.⁵ Clinically, lincomycin is mainly used to treat purulent inflammation and infection of the respiratory, digestive and urogenital system, as well as bone inflammation.^{6,7} After medication, common adverse reactions can manifest as abdominal pain, severe flatulence, diarrhea, nausea, vomiting and other gastrointestinal discomforts, as well as fever, thirst, fatigue and other symptoms.⁸ Occasionally, it can cause allergic reactions such as rashes and angioedema, and anaphylactic shock or even death in severe cases.^{9,10}

The reasons underlying the poisoning and death caused by antibiotics are different from common drugs. Due to the potential for allergic reactions, the route of administration, dosage and drug formulation are often contraindicated.¹¹ At present, clinical cases involving antibiotic poisoning and death are mainly caused by overdose or the particularly sensitive constitution of patients. During the last decade, there have been cases of poisoning or death caused by the use of lincomycin.¹² Previously common methods for the determination of lincosamides in medicines are based on high-performance liquid chromatography (HPLC) with UV detection at a wavelength range of 200–210 nm.^{13–15} Several studies have indicated that more selective methods, such as liquid chromatography-tandem mass spectrometry (LC-MS/MS), were applied for the determination of lincomycin or clindamycin in many matrices, such as animal meat, milk, eggs, honey, fish and environmental samples.^{16–18} However, a gap still exists in the current research about the determination of lincomycin in human whole blood or plasma. Only two LC-MS/MS method characterizations of lincomycin in blood samples have been published.^{19,20} As the popularity of LC-MS/MS has

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increased, its high sensitivity and fast detection speed have been unanimously recognized. At this point, it is necessary to develop a routine sample processing and instrument analytical methods for the quantitative analysis of lincomycin in human blood to assess allergy cases in clinical or forensic science.

In this study, a protein precipitation extraction (PPE) method was used to pretreat blood samples, and the lincomycin content in the blood was detected by the LC-MS/MS method. The validity and applicability of the analytical method was evaluated by the method validation system previously established,^{21–23} and it was applied to the quantitative analysis of lincomycin in human blood samples. The method for the routine monitoring of lincomycin contents in human blood was developed, fully validated, and successfully used in a forensic medical appraisal of lincomycin allergy fatal cases.

2. Materials and methods

2.1. Chemical and reagents

Lincomycin (100 mg, purity of 98.7%) was purchased from Cerilliant (Round Rock, USA) and lincomycin-D3 (100 µg/mL, purity ≥ 98%), used as internal standard (I.S.), was obtained from First Standard® (A Chemtek Inc., Worcester, USA). EDTA-K₂ (purity ≥ 98%) was purchased from CNW (Shanghai, China). Methanol and acetonitrile (HPLC grade) were both purchased from Merck (Darmstadt, Germany). Ammonium acetate (HPLC grade) and formic acid (analytical grade) were supplied by Fluka Chemical Co. (Bruchs, Switzerland). Ultra-purified water was filtered through a Milli-Q Advantage Elix Essential 3.5.10.15 system (Merck KGaA, Darmstadt, Germany). A stock standard solution (1 mg/mL) of lincomycin was prepared in methanol and stored in the dark at a temperature below 4 °C. The working solutions at level of 1.0 and 10.0 µg/mL were prepared daily when used.

2.2. Sample preparation

The blank human blood samples from different donors, which were provided by the Jining City Blood Station (Jining, China), had been tested and contained no drugs, whereas the test samples were the venous blood of the deceased suspected of lincomycin allergy. EDTA-K₂ was added as anticoagulant to all the samples before being stored in containers and kept frozen until processing. Thawed blood samples were homogenized using an MS3 Oribital Shakers (IKA, Staufen, Germany) and an accurate amount of 100 µL of each were transferred. First, after adding 10 µL of I.S. (lincomycin-D3, 10 µg/mL in methanol) to 100 µL of sample, the blood sample was diluted by 900 µL deionized water. Next, 1 mL of methanol was used for extraction through a 20-min vibration (EYELA, Tokyo Rikakikai Co., Ltd, Tokyo, Japan) and 10 min of ultrasonic treatment (SBL-15DT, Scient Z, Ningbo, China). Then the samples were centrifuged for 10 min at 4000 rpm (5804R, Eppendorf, Hamburg, Germany), and 2 µL of the supernatant that was filtered by a 0.22 µm syringe filter was injected into the LC-MS/MS system.

2.3. Liquid Chromatography-Tandem Mass Spectrometry

All experiments were carried out on a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) and on a 5500QTRAP mass spectrometer (Applied Biosystems Sciex, Foster City, CA, USA) with an electrospray Turbo spray interface. All data were acquired and processed using Analyst 1.6.3 and MultiQuant 3.0.2 Software.

The HPLC analysis was performed at a temperature of 35 °C on a Kinetex® Biphenyl 100 Å column (100 × 3.0 mm, 2.6 µm, Phenomenex, Torrance, USA). Experiments used 5 mmol/L of an ammonium acetate buffer mixed with 0.1% formic acid as eluent A and acetonitrile as eluent B, at a flow rate of 0.3 mL/min. The injection volume was set at 2 µL. The elution program started at an initial composition of 10% B and increased to 30% in 3 min, then eluent B was ramped to 90% in 5 min and held for

2 min before returned to the starting conditions in 9 min.

The MS/MS instrument system was operated using electrospray ionization (ESI) in the positive mode. The detection conditions were optimized previously to afford the highest relative intensity: an ion spray (IS) voltage of 5500 V and a source temperature of 550 °C were applied. The curtain gas (CUR) was 30 psi, the ion source gas 1 (GS1) was 45 psi and the ion source gas 2 (GS2) was 30 psi. The method was utilized in the multiple reaction monitoring (MRM) mode. Precursor ions, product ions, retention times (Rt) and the optimum turning parameters (de-clustering potential (DP), collision energy (CE) and collision cell exit potential (CXP)) for compounds are shown in Table 1.

2.4. Method validation

The described method was validated according to Peters et al.²¹, Matuszewski et al.²² and Meng et al.²³ Quality assurance of the developed method was evaluated by measuring parameters such as selectivity, specificity, the limit of detection (LOD), the limit of quantification (LOQ), linearity, accuracy, precision, stability, carryover, recovery and matrix effect.

Selectivity was tested in ten different blank blood samples collected from healthy donors to analyze whether the endogenous substances in the whole blood interfered with the detection of lincomycin.

Specificity was confirmed on the basis of spiked samples (n = 6) from different sources. The spiked blood samples were mixed with 10 µL of lincomycin and 10 µL of I.S. working solutions, both solutions at a concentration of 1 µg/mL. The determination was to evaluate the applicability of this method to detect lincomycin in blood by analyzing the peak shape, ion current intensity of the lincomycin characteristic ions, and the separation effect with I.S.

The LOD was based on the peak-to-peak noise of the baseline near the analyte peak obtained by an analysis of spiked samples and on the minimal value of a signal-to-noise ratio of 3 (S/N ≥ 3). The LOQ was calculated for the compound by a signal-to-noise ratio of 10 (S/N ≥ 10) in spiked samples. Moreover, an accuracy and precision within ±20% was required for the LOQ.

The linearity of the method was checked on the basis of calibration curves using spiked blank blood samples. The linear calibration range used was from 5 to 100 ng/mL, with seven concentration levels. Peak signal ratios between the analyte and I.S. were plotted against the concentration of lincomycin, and a linear regression was performed.

The method accuracy and precision were evaluated at three concentrations (5, 20, 80 ng/mL) within the linear range. The accuracy was calculated as the percent deviation between the mean calculated concentration at each level and the corresponding spiked concentration by six replicates. The precision was calculated as the relative standard deviation (RSD%) between six replicates during the same day (intraday precision) and for three consecutive days (inter-day precision). The criteria of accuracy ranged from 85% to 115% (80%–120% near the LOQ), and precision was within 15% RSD (20% near the LOQ).

The freeze-thaw stability was evaluated spiked blood samples containing 5, 20, 80 ng/mL of lincomycin (n = 6). Following an initial freezing period of 24 h (−20 °C), the samples were thawed for 12 h (to 25 °C) before determination. Stability verification was performed for three cycles of freeze and thaw. By comparing the repeated freeze-thaw

Table 1
MRM parameters and retention times of compounds.

Compound	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (eV)	CXP (V)	Rt (min)
Lincomycin	407.3	126.1 ^(a)	30	36	15	4.02
	407.3	359.1	50	24	10	
Lincomycin-D3	410.0	129.1 ^(a)	30	35	16	4.01
	410.0	362.1	40	25	11	

^a Was used as quantitative ion.

samples with the control samples analyzed immediately after preparation, the average measured concentration of the processed samples should be within 80%–120% of the controls.

Carryover was assessed by placing one control blank (without analyte and I.S.) immediately after every maximum concentration sample (80 ng/mL) in each accuracy and precision run. It was acceptable if carryover blank had lincomycin peaks that were <20.0% of the lowest peak area of LOQ and carryover blank had IS peaks that were <5.00% of the mean peak area of IS in the run.

Recoveries (R) and matrix effects (ME) were determined by the analysis of spiked samples at three concentrations (5, 20, 80 ng/mL). The calculation formulas were as follows:

$$R = A/B \times 100\% \quad (1)$$

$$ME = (B/C - 1) \times 100\% \quad (2)$$

Where A was the concentration of spiked blood sample analyzed, B was estimated by the blank blood sample that was extracted and spiked with the analyte at the designated concentration, and C was the standard concentration in the methanol sample.

2.5. Quality assurance/quality control

A procedural blank (one laboratory blank, one control blank spiked with I.S. only, and one standard-spiked matrix sample) was run before samples to assess for potential interference and cross-contamination from the procedure. During the injection time, lincomycin was not detected in the procedural blanks. Three duplicate samples were set in each station, and lincomycin concentrations in parallel samples were consistent. The concentrations of lincomycin in blood samples were quantitatively determined by the internal standard method using peak area, which were kept consistent in parallel samples.

3. Results and discussion

3.1. Method optimization

A series of preliminary experiments with human blood samples was performed, testing different extraction methods. Methanol and acetonitrile were checked as solvent extractions, respectively, to compare the recoveries and peak shapes. The average recovery of protein precipitation by acetonitrile was 52.50%, while 76.63% was recovered using methanol as a solvent. Fig. 1 compares the chromatograms for the analyte using these two solvents. It indicates that the characteristic ion pair of lincomycin had more impure peaks when using acetonitrile as an injection solvent (Fig. 1-A). This phenomenon could be effectively improved after changing to methanol (Fig. 1-B). Therefore, PPE using methanol was selected because it provides a better recovery and peak shape for the target compound of interest.

3.2. Method validation

There were no compounds determined in the selectivity experiment, and the typical chromatograms of blank blood extract spiked with suitable lincomycin and I.S. in the specificity experiment are shown in Fig. 2. The migration times of lincomycin and I.S. were 4.02 and 4.01 min, respectively, with an ideal peak shape and ion current intensity. The results of these assays confirmed that there were no peaks of endogenous substances interfering with the determination of the analyte. At the same time, the addition of isotope I.S. would not interfere with the detection of lincomycin.

The LOD and LOQ for lincomycin are shown in Table 2. These values were low enough to determine the concentrations of lincomycin in human blood samples. The correlation coefficient was found to be more than 0.99, indicating good linearity in the concentration range of 5–100

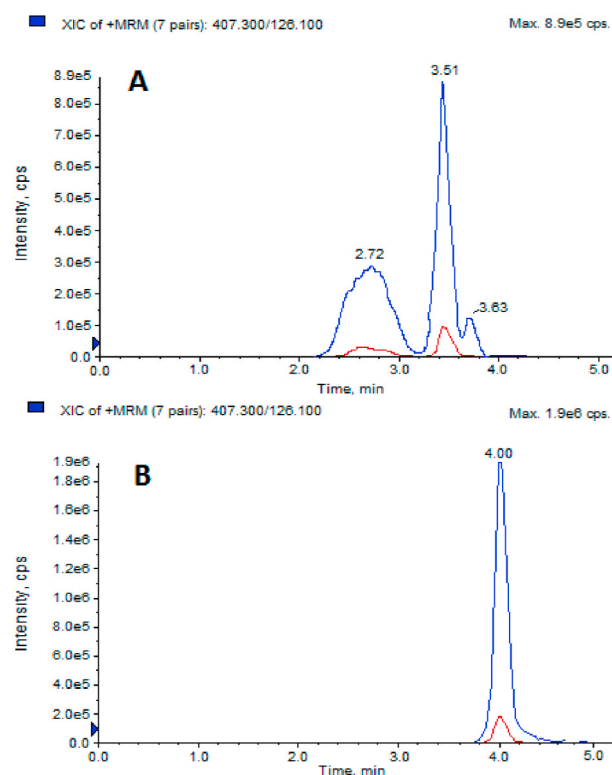


Fig. 1. The comparison of chromatograms for lincomycin using two different extraction solvents (A: acetonitrile; B: methanol).

ng/mL for lincomycin.

The method validation data are summarized in Table 3. The results showed that the accuracies of lincomycin were within $\pm 10\%$ at all concentration levels, whereas the overall precisions were <10%. These results confirmed that accuracy and precision values for the elaborated method are satisfactory for the determination of lincomycin. The average measured concentrations of the freeze-thaw samples were within 90%–100% of the controls, showing a stability of lincomycin in whole blood. Carryover wasn't obviously observed during method development, which peak areas were 0.0033% of lincomycin ($S/N < 1.0$) and 0.0042% of I.S. ($S/N < 1.5$) in carryover blank samples. The recoveries obtained with spiked experiments in the blood samples were >70%, indicating the method's good extraction efficiency. And the matrix effects at the three concentrations were within $\pm 15\%$, showing that the blood matrix had no obvious influence on the detection. It was therefore expected that the extraction and instrument parameter of the method were suitable for the identification and quantification of lincomycin in authentic blood samples of allergy cases.

3.3. Application to authentic cases

The developed methods of methanol-PPE and LC-MS/MS were applied to the analysis of human blood in authentic allergy cases. The determined person died of a suspected lincomycin allergy. After intravenous injection of lincomycin in May 2020, he felt unwell and eventually died after rescue. The deceased had mild laryngeal edema and lung bronchospasm accompanied by submucosal mast cell degranulation and eosinophil infiltration, with pulmonary congestion, pulmonary interstitial edema and other organizational changes, based on autopsy studies and other histopathological observations (Fig. 3). The above description is consistent with the pathological changes caused by allergic reactions.²⁴ Subsequently, the venous blood of the deceased was drawn within 2 days after admission and analyzed by the method established in this study. The analysis results showed that lincomycin

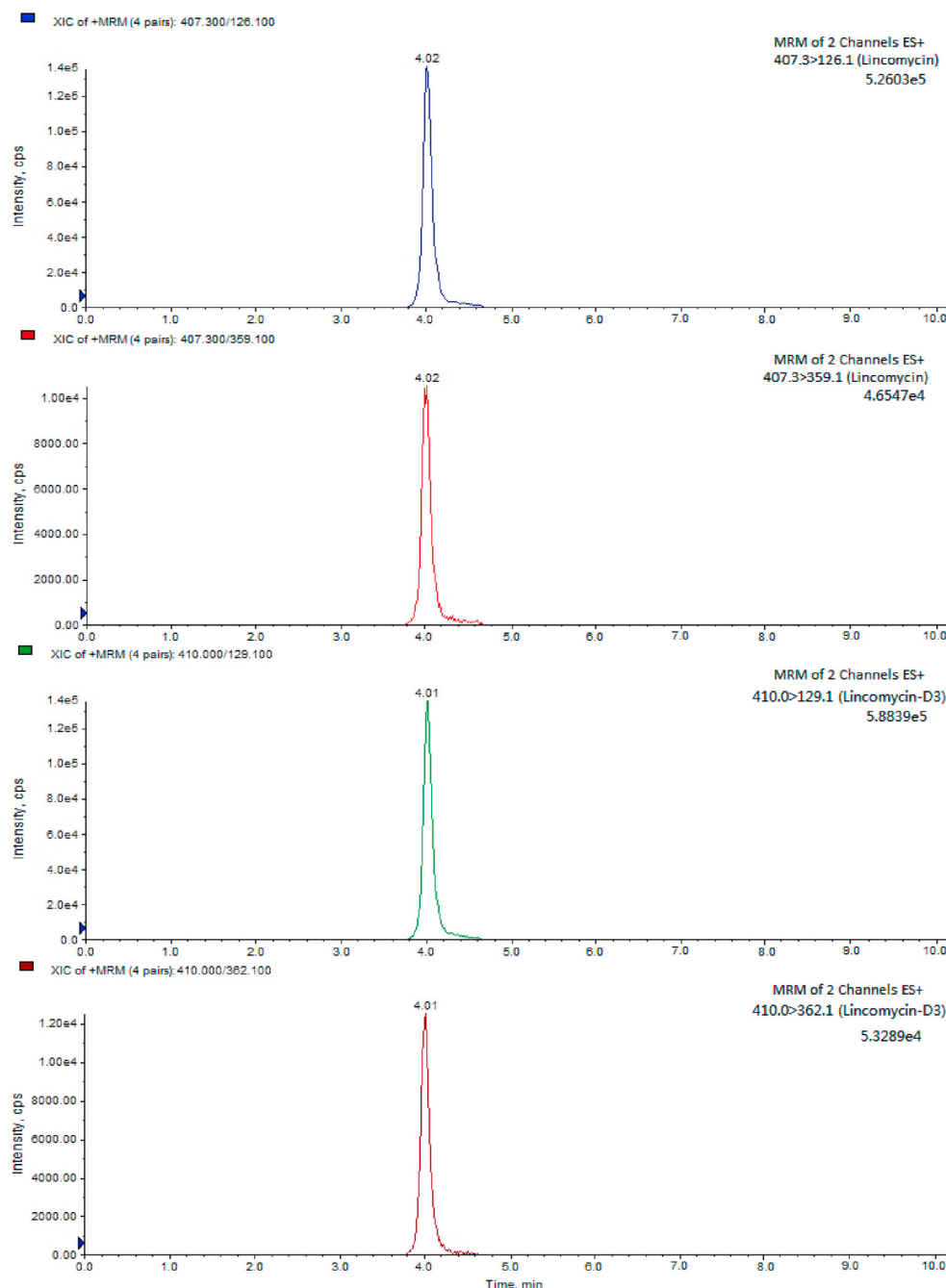


Fig. 2. Chromatograms of lincomycin and I.S. in spiked human blood samples (10 ng/mL).

Table 2

Linearity and method detection limits of lincomycin.

Compound	Linear equation	correlation coefficient	LOD (ng/mL)	LOQ (ng/mL)
Lincomycin	$y = 0.09508x + 0.23085$	0.99432	0.2	1.0

was detected in the venous blood of the deceased at 877 ng/mL, with the RSD of 4.45%. In summary, the study concluded that the deceased died of anaphylactic shock after the injection of the drug lincomycin.

Previous clinical cases have shown that the use of lincomycin can cause a series of adverse reactions, including allergic reactions (anaphylactic shock and rash, etc.), urinary system reactions (hematuria, urinary retention, etc.), nervous system reactions (dysgeusia),

hyponatremia and so on.^{20,25} Excessive doses, high concentrations, and rapid infusion rates of lincomycin can cause anaphylactic shock and lead to multiple organ failure and respiratory muscle paralysis. Ding et al. (2015) reported 31 deaths caused by the intravenous injection of lincomycin for the treatment of respiratory infections, bronchial inflammation and other diseases.¹⁰ The research results showed that for patients with a history of drug allergies (penicillin, cephalosporin, etc.), direct intravenous injection of lincomycin without a skin test posed a greater risk of sensitization. Previous studies have pointed out that the sensitization mechanism of lincomycin is similar to that of antibiotics such as penicillin, cephalosporins, sulfonamides, ciprofloxacin and tetracycline. Such drugs can react with IgG or IgM antibodies to generate soluble immune complexes, thereby mediating immune complex hypersensitivity reactions.^{26,27} The deposition of immune complexes in tissues results in a tissue reaction initiated by complement activation

Table 3

Accuracy, precision, recovery and matrix effect for lincomycin in spiked human blood samples.

Compound	Concentration (ng/mL)	Stability (%)	Accuracy (%) (n = 6)	Precision RSD (%)		Recovery (%) (n = 6)	Matrix effect (%) (n = 6)
				Intraday (n = 6)	Inter-day (n = 18)		
Lincomycin	5	98.02	92.82	5.53	6.40	72.70	-13.05
	20	95.98	100.40	1.64	2.21	84.13	-9.16
	80	96.76	97.25	1.19	2.45	73.06	6.73

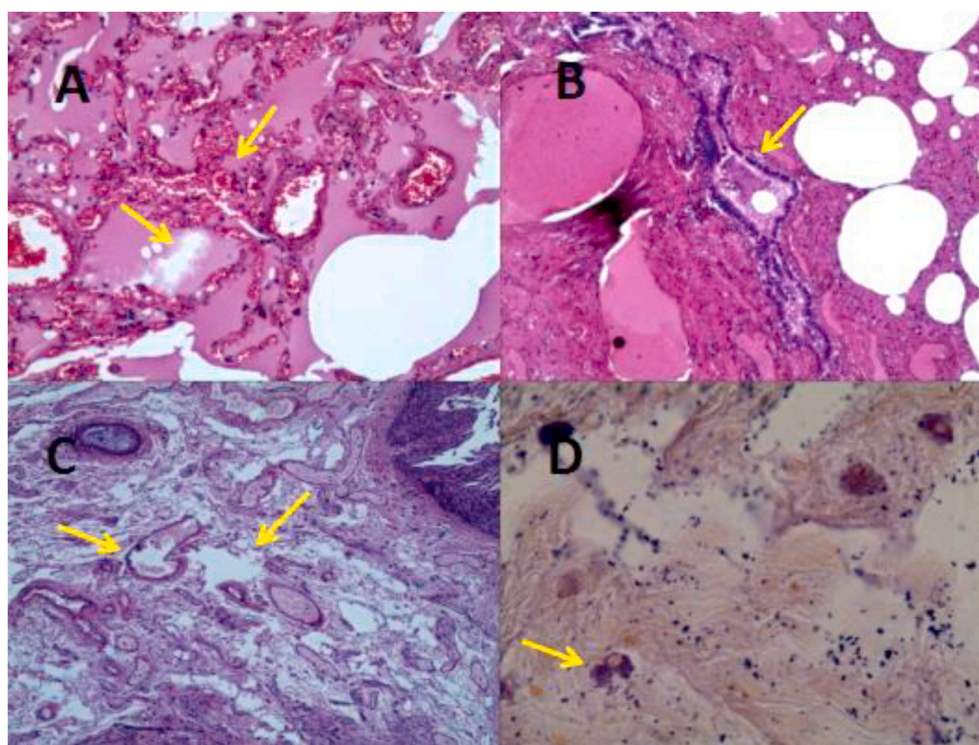


Fig. 3. The pathological sections of the deceased with allergic reactions to lincomycin (A: Extensive expansion and congestion of blood vessels in the lungs, edema fluid in most of the alveolar cavity; B: Part of bronchospasm showed petal-like changes; C: Dilation and congestion of blood vessels in the submucosal stroma of the larynx with loose and edematous histocyte; D: A large number of basophils and partial degranulation were seen in the interstitium of the larynx).

that may lead to mass cell degranulation, leukocyte chemotaxis, and inflammation induced by the cell influx. Angioedema may be seen, and arthritis or arthralgia and gastrointestinal symptoms of cramping, nausea, vomiting, or diarrhea occur in up to 67% of patients.²⁸ Although most reactions are mild and usually resolve spontaneously within a few days or weeks after discontinuing the drug, there are still a minority of severe allergic reactions in patients. The PPE combined with the LC-MS/MS method established in this study can accurately determine the content of lincomycin in human blood with the advantages of simple sample processing steps, fast analysis speed, high sensitivity, and good reproducibility. This method can be used for the analysis and verification of sporadic lincomycin allergy fatal cases in clinical and forensic science.

4. Conclusion

As a commonly used antibacterial antibiotic in clinical settings, the use of lincomycin can cause a series of adverse reactions and lead to anaphylactic shock, or even death, in severe cases. We provide a rapid and sensitive LC-MS/MS method for the detection of lincomycin in human blood samples. The method has simple pre-processing, high sensitivity and good selectivity, and is suitable for the detection and analysis of actual samples. In the authentic case application, the established method was used to detect and analyze the suspected allergic death case caused by lincomycin injection. Combined with the

pathological test results, it was confirmed that the deceased died of an allergic reaction to lincomycin. The research results can provide method references for the clinical and forensic identification of lincomycin allergy and death cases.

CRediT author contribution statement

Le Du: Writing - original draft, Methodology. Guangyan Li: Investigation, Resources. Wenjing Gong: Writing - review & editing, Formal analysis, Funding acquisition. Jun Zhu: Project administration, Supervision. Li Liu: Data curation. Lei Zhu: Software. Zengjia Liu: Validation.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Ethical permission was granted through the Science and Ethics Committee of Jining Medical University. Informed consent was obtained from each individual's family in this study.

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Declaration of competing InterestCOI

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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